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Interaction du virus de Schmallenberg et des virus apparentés dans des cellules de mammifères et d'insectes vecteurs

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UNIVERSITE DE NAMUR

Faculté des Sciences

**Interaction du virus de Schmallenberg et des virus apparentés dans des
cellules de mammifères et d'insectes vecteurs**

**Mémoire présenté pour l'obtention
du grade académique de master en biochimie et biologie moléculaire et cellulaire**

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Janvier 2015

Interaction du virus de Schmallerberg et des virus apparentés dans des cellules de mammifères et d'insectes vecteurs

DEPIERREUX Delphine

Résumé

Le virus de Schmallerberg (SBV) a été découvert en Allemagne en 2011, après de graves lésions qu'il causa chez les fœtus d'ovins et de bovins. Il fait partie de la famille des Bunyaviridae, genre Orthobunyaviridae, séroroupe Simbu (Hoffmann *et al.*, 2012). Il possède un génome tri-segmenté (S, M, L) et est transmis par des *Culicoides* (arthropodes). SBV pourrait être apparu suite à un réassortiment entre les virus Sathuperi et Shamonda (Yanase *et al.* 2012). SBV présente un cycle de vie en alternance entre vertébrés et insectes. Afin de connaître le comportement de ce virus au sein de ces 2 hôtes, des infections *in vitro* de cellules insectes (KC) et mammifères (BHK-21) ont été réalisées. Il a été constaté que l'infection par SBV de cellules BHK-21 était aigue et menait à la mort cellulaire alors que l'infection de cellules KC était persistante et ne menait pas à des effets cytopathogènes. Le faible taux de réplication viral en cellules KC pourrait être dû à une réponse RNAi ou à une séquestration de protéines virales importantes pour la réplication de SBV. Un mécanisme n'impliquant pas la rupture de la membrane cellulaire des KC pourrait expliquer l'absence d'effets cytopathogènes dans cette lignée cellulaire. Afin de tester ces deux dernières hypothèses, des anticorps dirigés contre SBV ont été produits pour pouvoir suivre sa réplication au sein des cellules. La nucléoprotéine N de SBV a été choisie comme cible en raison de son niveau d'expression élevé dans les cellules infectées. Elle a été synthétisée en collaboration avec BioX et caractérisée par gel 1D, spectrométrie de masse et gel 2D. Les poly-sérums de 2 lapins immunisés avec cette protéine recombinante ont été étudiés par ELISA, Western blot et immunofluorescence. Les anticorps produits étaient spécifiques, sensibles, et capables de reconnaître la nucléoprotéine sous forme recombinante et native. Cependant, ils n'ont pas pu mettre en évidence des KC infectées probablement en raison du faible taux d'amplification de SBV. Dès lors, une souche virale présentant un meilleur taux d'amplification en KC était nécessaire. Dans ce but, une infection à long terme en cellules KC a été réalisée. L'évolution de l'amplification virale au cours du temps a été suivie par PCR quantitative et par titrages. Après 5 passages cellulaires successifs, une augmentation importante du taux de réplication a été observée, suggérant une adaptation virale. Pour connaître les raisons de cette adaptation, le segment S et la région hypervariable du segment M de SBV ont été séquencés. Trois mutations sont apparues dans la région hypervariable et se sont maintenues à travers les différents passages cellulaires étudiés. Ce travail fournit les outils nécessaires à la réalisation de co-infections en cellules KC impliquant SHAV et SATV. Celles-ci nous permettront d'étudier le réassortiment de ces 2 virus et *in fine* d'en savoir plus quant à l'origine de SBV.

Mémoire de master en biochimie et biologie moléculaire et cellulaire

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Delphine

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Introduction

Introduction

Context

During the last year, the rise and the re-emergence of infectious disease such as bluetongue, SARS or Ebola generated a keen interest around viruses. However, virology is a relatively new science and there is still much to study and to understand.

Indeed, the concept of viruses was established in the period of 1886 to 1903 thanks to the work of Adolf Mayer, Dimitri Ivanofsky and Martinus Beijerinck on Tobacco mosaic virus (Fields virology, 2007). In 1939, electronic microscopy was developed and the first virus to be observed was the Tobacco mosaic virus. Thereafter, the development of cell culture, the discovery of nucleic acids (DNA and RNA) and the improvement of molecular biology techniques permit to learn more about their biology. Since the beginning of virology, many viruses have been discovered in organisms such as plants, vertebrates, bacteria, yeast... With the discovery of these viruses, arose the question of their origin. Three main hypotheses have been proposed (Wessner, 2010).

- The progressive hypothesis: viruses represent genetic elements that gained the ability to move between cells.
- The regressive hypothesis: viruses represent previously free-living organisms that became parasites.
- The virus-first hypothesis: viruses predate/co-evolve with their present cellular hosts

As viruses represent a very diverse group, it is not possible to choose one of these three hypotheses. Retroviruses match with the progressive hypotheses, while DNA viruses match with the regressive hypothesis. Perhaps viruses arose via multiple mechanisms or via an uncovered mechanism.

If a stringent definition of a living organism is considered, viruses cannot be considered as living. According to Andre Lwoff, 1957: "*Viruses are corpuscular biologic objects, endowed with genetic continuity through a DNA or RNA constituting their genome. They lack enzymatic system capable of ensuring their synthesis, therefore they have to borrow bioenergetics machinery of the infected cell to replicate them in huge copy number*". This definition made them hard to be related to the tree of life and classified. However, viruses are listed following taxonomy similar to those of living organisms: Order-Family-Subfamily-Genus-Species. In addition, viruses can be separated following the nature of the viral genome. There are DNA or RNA viruses which are simple or double stranded. For RNA single stranded viruses, there are positive or negative polarities and there are segmented or non-segmented viruses.

Despite their diversities, viruses share several common features. They are small, generally less than 200 nm (Wessner, 2010), they cannot produce ATP (Adenosine Tri Phosphate) and they do not have ribosomes. These characteristics made them obligatory intracellular organisms.

So, to viruses, they need hosts and sometimes vectors to ensure the viral transmission from one host to the other. Therefore, viruses are subjected to different immune pressure in the different hosts and the vectors they infect. To counteract the antiviral response raised against them, viruses need to evolve rapidly. There are four main mechanisms by which they modify their genome:

- Mutation

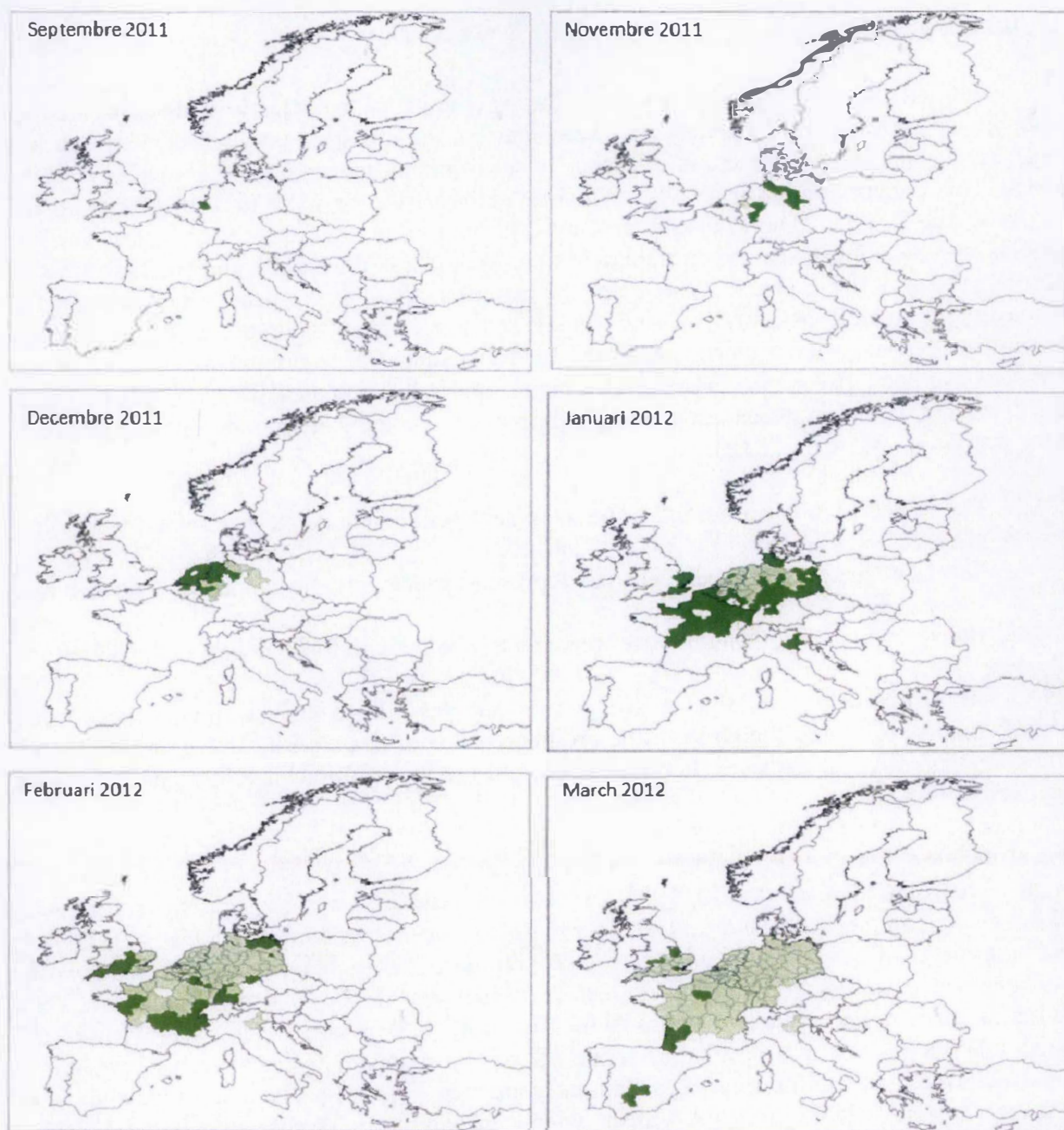


Figure 1 : Representation through time of territories where SBV infected ruminants have been detected between September 2011 and March 2012, showing SBV outbreaks through Europe. In dark green, new territories concerned by SBV infections in herds compared to previous date, in light green previous territories concerned by SBV infections on herds (EFSA report, 2012).

- Reassortment
- Recombination

The relative importance of each of these mechanisms varies between viruses according to the nature and structure of their genome. However, mutations are so frequent that there are no ssRNA viruses that are replicated without a mistake in their genome. So we do not talk about viruses as individuals but rather as a population, more precisely as quasi-species. A Quasi-specie is a population of variant viruses in relative equilibrium with the environment, but capable of swift adaptation because of a high spontaneous mutation rate (Fields virology, 2007). Every year, new viruses are discovered and questions about their origin, their mode of transmission, their pathogenicity and their structure are generated, thus continuing the exciting story of viruses.

Schmallenberg virus (SBV) is an example of new viruses that emerged during the last five years and which has a medical relevance. This work will focus on SBV and its two related viruses, more precisely about their interactions with their vectors the *Culicoides*. Before going into the details let us introduce the main players, in the first part Schmallenberg virus and in the second part, *Culicoides*.

Schmallenberg virus

Discovery

At the end of the summer 2011, diarrhea, hyperthermia and drop of milk production in adult dairy cows were reported in North-West Germany and Netherlands. In December 2011, abortion and malformations were detected in calf and lambs born from females infected during their gestations (Beer *et al.*, 2012). After the exclusion of well-known pathogens, the Friedrich Loeffler Institute (FLI) identified viral RNA sequences in blood samples from symptomatic cattle in November 2011 (Hoffmann, 2012). A bio-informatic analysis revealed that these RNA sequences were closely related to two ruminant viruses that have never been isolated in Europe. The sequence combination was unique and had never been reported before. This new virus was named Schmallenberg virus (SBV) after the city where it was primarily isolated.

SBV was then reported in 2012 in several countries in Western Europe and it was showed that SBV has spread rapidly over large parts of Europe (Figure 1). Today, we can consider that all Europe is virtually affected by SBV.

Host range

Since the detection of SBV in sheep, goats and cattle in 2011, antibodies raised against SBV have been detected in red deer, roe deer (Linden *et al.*, 2012) but also in dogs (Wensman *et al.*, 2013), bison, moose, alpacas, and buffalos (EFSA, 2013). Up to now, the host range seems to be limited to the previously described animals and there is no evidence for zoonotic transmission (Reusken *et al.*, 2012). The presence of SBV antibodies was investigated in persons living or working in farms where SBV has been suspected. The serums of 301 people were tested. There was no evidence either of SBV-neutralizing antibodies or the presence of any viral genome sequences in them (Reusken *et al.*, 2012).

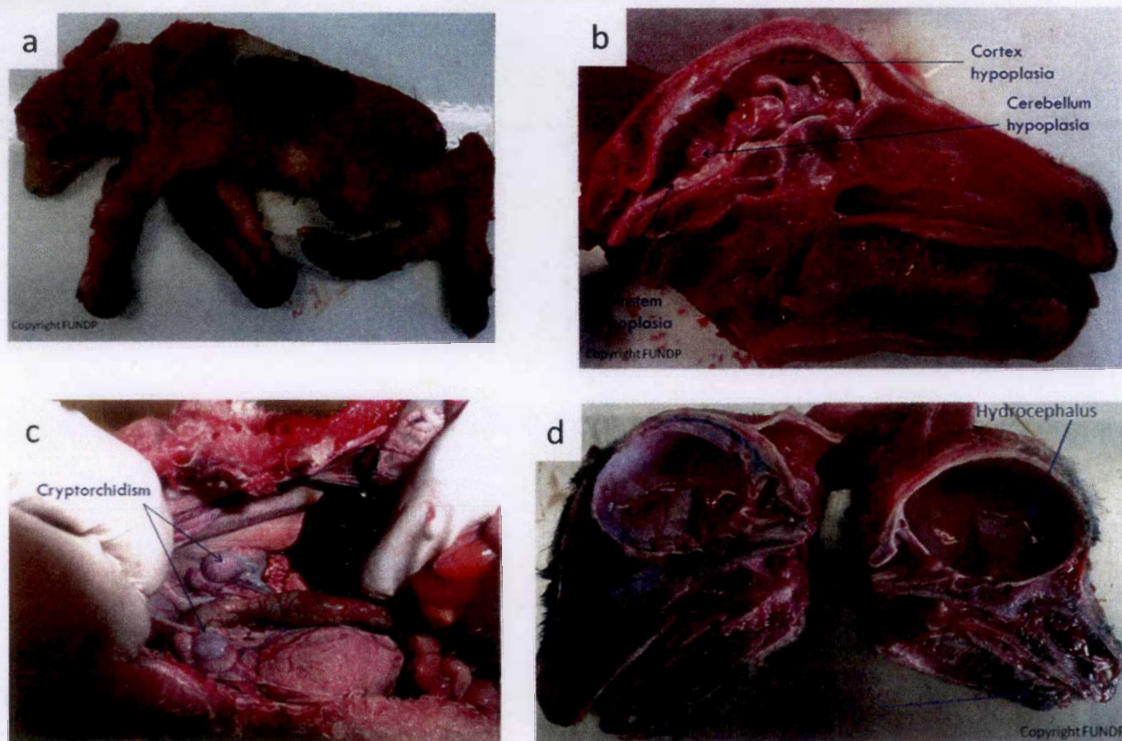


Figure 2 : Lesions formed in lambs infected with SBV at critical development stages. A: external lesions consisting in scoliosis and arthrogryposis. **B:** nervous system lesions; Cortex hypoplasia, cerebellum hypoplasia and brainstem hypoplasia. **C:** cryptorchidism **D:** hydrocephalus and brachynathia.

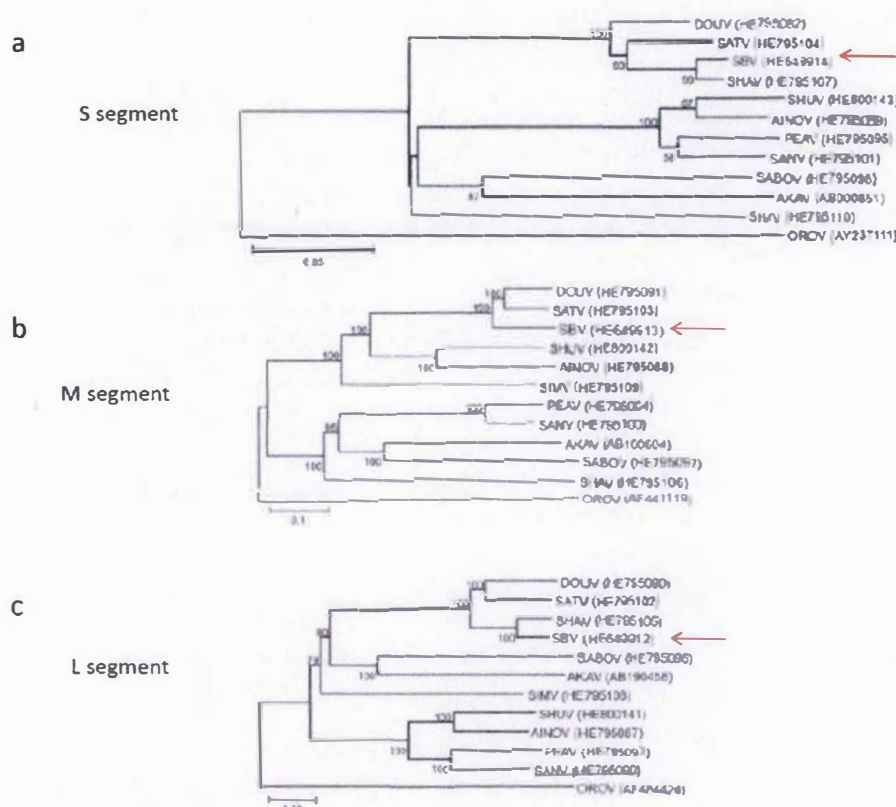


Figure 3 : Phylogenetic tree of Simbu serogroup viruses for the S , M and L coding regions. We can see proximity between Shamonda, Sathuperi and Schmallenberg viruses for each segment. GenBank accession numbers are shown in parenthesis. Numbers at nodes indicate percentage of 1,000 bootstrap replicates. Scale bars indicate nucleotide substitutions per site. DOUV, Douglas virus; SATV, Sathuperi virus; SBV, Schmallenberg virus; SHUV, Shuni virus; AINOV, Aino virus; SIMV, Simbu virus; PEAV, Peaton virus; SANV, Sango virus; AKAV, Akabane virus; SABOV, Sabo virus; SHAV, Shamonda virus; OROV, Oropouche virus. ND, not determined. (Goller *et al.*, 2012)

Symptoms

Clinical signs can be separated in two main classes: those observed in the offspring of infected females and those observed in adults.

Symptoms are more acute in adult cattle than in sheep and goats, and include hyperthermia, diarrhea, anorexia and drop of milk production until 50% (Muskens *et al.*, 2012). Symptoms last 2 to 6 days in cattle (Hoffman *et al.*, 2012 cited in Doceul *et al.*, 2013).

Symptoms in the offspring of infected females consist in abortions and malformations and were reported in calves, lambs and goatskins. It was established that if SBV infects serologically naive ruminants, it crosses the placenta and replicates in the fetus, leading to arthrogryposis, cryptorchidism and abnormalities of the central nervous system (CNS) (Garigliany *et al.*, 2012) (Figure 2). As Akabane virus causes similar lesions and presents a related genomic sequence to SBV, it was postulated that SBV and Akabane could have similar pathogenic mechanism. So, based on the data available for Akabane virus, it was hypothesized that SBV needs to infect the mother during a precise period of time to lead to abortions and malformations in offspring: between 60 and 180 days for cows (Martinelle *et al.*, 2012) and between the 30 and 50 days of gestation for sheep (Martinelle *et al.*, 2012).

The infection window which leads to these lesions could be explained by two terminals. The first terminal is the time needed for the formation of placentomes which enable mother-foetus exchanges. Before their formation, SBV does not have access to the fetus. The second terminal is the time needed to form the blood brain barrier which limits the access to the fetus CNS. After its formation SBV does not have any access to the fetus CNS (Varela *et al.*, 2013).

SBV presents a neurotropism (Varela *et al.*, 2013) that can explain the serious lesions of the CNS as well as dysfunction of several hypothalamo-hypophyso-glandular axes. These last could lead to cryptorchidism as well as other disorders encountered in SBV-infected fetuses. Arthrogryposis would have a neurogenic origin (Mayhew I.G., 1984 cited in Martinelle *et al.*, 2012)).

Genome and function of the virus encoded proteins

Analysis of the viral genomic sequences revealed that SBV was related to Akabane, Shamonda (SHAV), Aino, Douglas and Sathuperi (SATV) viruses that belong to the *Bunyaviridae* family, the *Orthobunyavirus* genus and the *Simbu* serogroup (Hoffmann *et al.*, 2012) (Figure 3). Viruses belonging to the *Bunyaviridae* family produce membrane-enveloped spherical virions of 100 nm and possess a tripartite single stranded negative RNA genome.

For SBV, coding capacities have been predicted to be similar with other orthobunyaviruses. SBV genome encodes 6 proteins: two non-structural (NSm and NSs), two internal structural proteins (N nucleoprotein and L polymerase), and two external structural proteins (glycoprotein N and glycoprotein C). The coding sequence of these proteins is distributed on three negative single-stranded RNA segments; long (L), medium (M) and short (S) (Figure 4) (Doceul *et al.*, 2013).

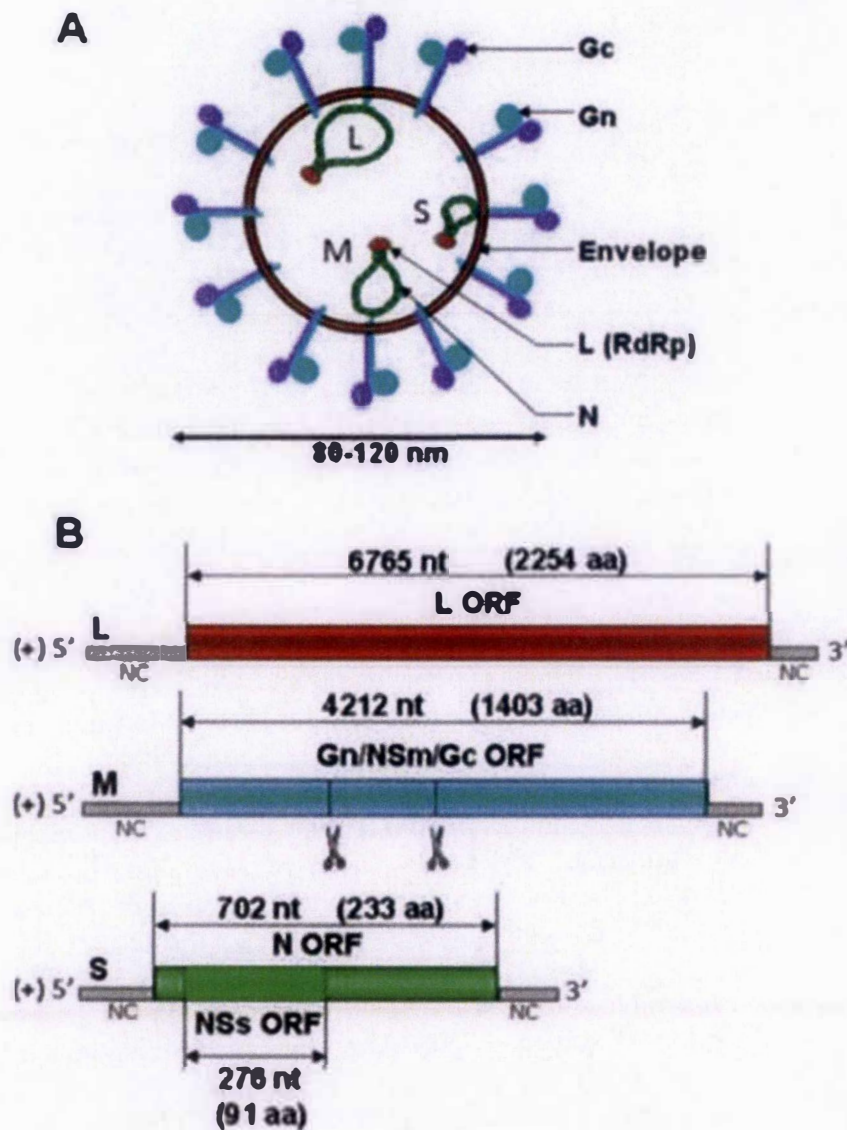


Figure 4: Schmallenberg virus structure and genome. A: representation of SBV structure and its average diameter (80-120 nm) determined by electron microscopy. Gc and Gn are surface glycoproteins, L RdRp is the viral polymerase and N nucleoproteins bound and protect RNA, envelope is derived from Golgi membrane of the host cell. B: representation of the three RNA genome segment of SBV, their length, and the proteins they code for. L segment codes for L viral polymerase, M segment codes for both Gn and Gc glycoproteins and non structural M protein, S segment codes for N nucleoprotein and non structural S protein (Doceul *et al.*, 2013).

L segment (6882 nt) encodes the RNA-dependent RNA polymerase (RdRp), also called L polymerase. L polymerase has to recognize promoter regions to initiate replication and transcription to produce messenger RNA, genomic RNA and complementary RNA. These RNAs do not possess a poly A signal which is normally used as a signal to stop the transcription. So, L polymerase stops the transcription by another mechanism. It has been postulated that L polymerase recognizes secondary structures and termination signals. For Simbuviruses, secondary structure consists of highly structured hairpin at the end of the segments and prevents dsRNA formation when antigenomic RNA is synthesized. Thus, counteracting antiviral response since dsRNA is a pattern that triggers antiviral response in cells (Coupeau *et al.*, 2013).

M segment (4373 nt) encodes a precursor polyprotein that is further cleaved into the envelope glycoproteins Gn/Gc (glycoprotein N-terminal and glycoprotein C-terminal) and the non-structural protein NSm (Figure 4). This segment also contains a hyper-variable region situated between NSm and Gc ORF in the N-terminal region that explained the variation of the Gc glycoprotein (Coupeau *et al.*, 2013). Gc and Gn heterodimers recognize cellular receptors and are the major targets of the immune response raised against the viral particle. They also play a role in virus assembly and budding by interacting with each other, with host proteins and the nucleocapsid (Briese *et al.*, 2013), mainly by their cytoplasmic tail regions (Shi *et al.*, 2007). The NSm protein is thought to play a role in virus assembly (Shi *et al.*, 2006), since it has been observed in Golgi derived viral factories and since its mutations combined with Nss mutation impairs viral production (Kraatz *et al.*, 2014). However, this effect on viral replication is specific to double mutants since NSm single mutant replicated to comparable level as the parental SBV (Kraatz *et al.*, 2014). Moreover, NSm has been implicated in virulence, its mutation in IFNAR-/- mice lead to less virulent phenotype (Kraatz *et al.*, 2014).

S segment (839 nt) encodes the nucleoprotein N and the non-structural protein NSs and L polymerase uses shift of the reading frame to produce these both proteins (Doceul *et al.*, 2013).

SBV N nucleoprotein is the most abundant protein in SBV and in SBV infected cells. N nucleoprotein interacts with viral genomic and antigenomic (replicative intermediate) RNAs to wrap them with multiple copies of N to form complexes called ribonucleoprotein (RNPs). This association is critical for replication and transcription (Dong *et al.*, 2013) by the viral RNA-dependant RNA polymerase (RdRP). RNPs also protect the viral genome from ribonucleases and help to avoid triggering the host innate immune response. Indeed, ssRNA can be recognized by TLR7 and TLR8 which trigger IFN-1 and TNF-alpha production. There is a highly positive charged cleft between the N- and C-terminal domains, which could be the genomic RNA binding site. Therefore, it is easy to explain how the viral genomic RNAs are protected by nucleoproteins, as the RNA segment (negatively charged) is wrapped inside their positively charged ring structures. However, it is more difficult to explain how the RNA, in the form of the RNP, can be accessed by the RNA-dependent RNA polymerase (L protein).

According to Dong *et al.*, 2013, for SBV N nucleoprotein there is a third arrangement, in which half of the protomers keep the RNA binding clefts inside the oligomers and half of the protomers expose the RNA binding clefts outside the oligomers. This arrangement may be an intermediate that indicates how the genomic RNAs are protected and how the RNA can be accessed in the RNP template for replication and transcription. The N protomer consists in two domains, N- and C-terminal domain and two flexible arms, N- and C-terminal arm. The

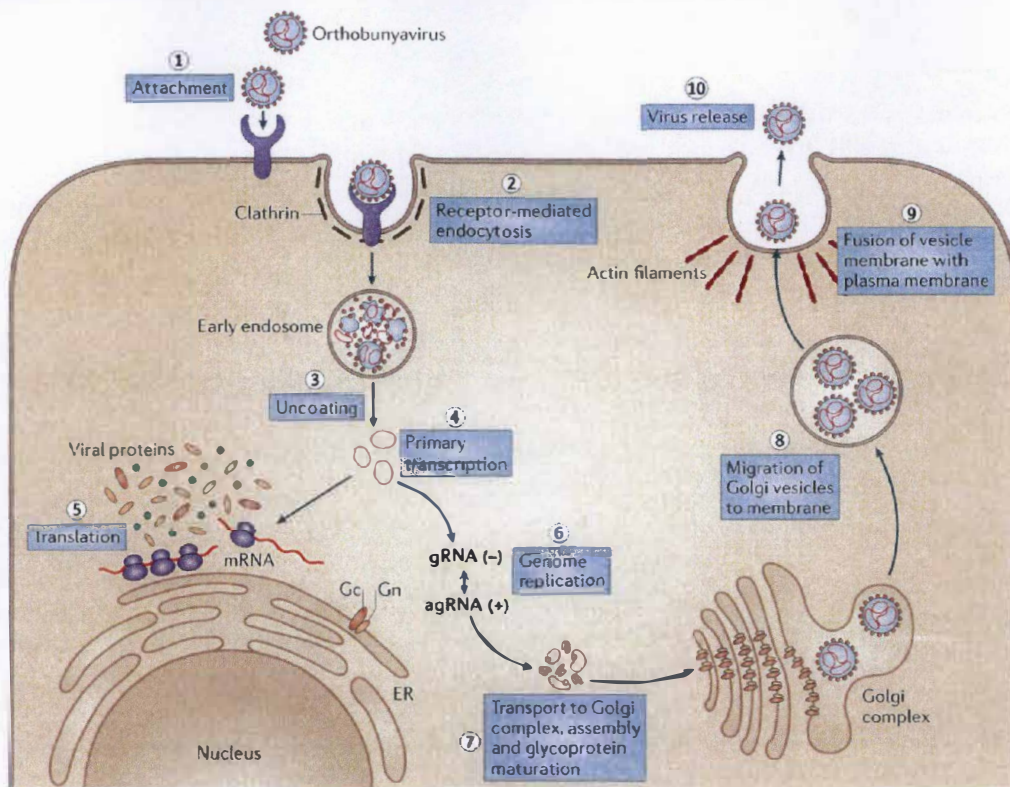


Figure 5: Orthobunyavirus replication cycle. Replication begins with viral attachment to host receptors (1), which is mediated by viral glycoproteins Gc and Gn. Then, virus is internalized via receptor-mediated endocytosis (2). Acidification leads to peptide conformational changes and to membranes fusion (3). Once viral RNA genome is in the cytoplasm, primary transcription occurs (4), leading to mRNA production that are subsequently translated into viral proteins (5) or replicated to produce genome copies (6). These last are transported to Golgi, assembled with viral proteins including glycoproteins that will complete their maturations in the Golgi. Then, viral particles bud from the Golgi and migrate to the plasma membrane (8) where both membranes fuse (9) leading to virions release in the extracellular environment (10). (Elliott, 2014)

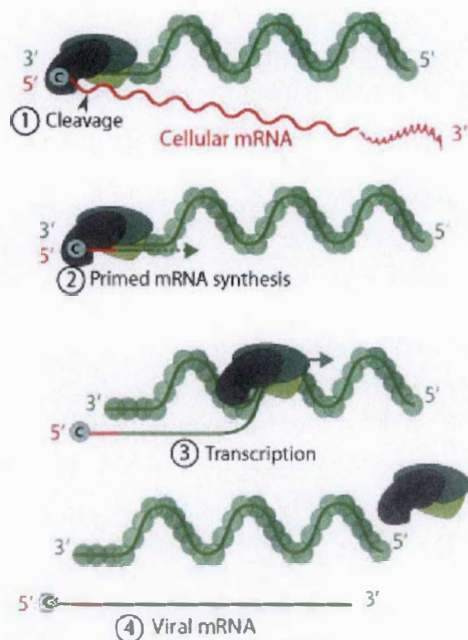


Figure 6: Illustration of cap-snatching process. First, the viral L polymerase recognizes a cellular mRNA and the termini of an RNA segment. Then, the polymerase cleaves the cellular mRNA and uses it as a primer to start the transcription. It is important to note that the tripartite polymerase structure on that illustration has not been verified for SBV and is influenza inspired (inspired from http://viralzone.expasy.org/all_by_protein/839.html consulted on 28/10/2014.)

nucleoprotein purified under native conditions forms a tetramer, while the nucleoprotein obtained by denaturation and refolding process forms a hexamer. Both N-terminal and C-terminal arms are involved in N-N interaction and oligomerization (Dong *et al.*, 2013).

The second protein coded by S segment, the NSs protein, is implicated in blocking the host interferon response (Barry *et al.*, 2014 and Kraatz *et al.*, 2014). Indeed, mutants with a NSs-deletion induced high levels of interferon and showed a growth defect in sheep SFT-R cells. In addition, it has been showed by Barry *et al.*, (2014) that SBV NSs protein shuts off protein synthesis in targeting a RNA polymerase II subunit RPB1 for degradation. Therefore, transcription is blocked leading to a decrease of IFN-1 synthesis and the interferon response. This contributes to counteract the antiviral response to SBV infection. Moreover it has also been described that SBV NSs protein enhances apoptosis in mammalian cells suggesting that NSs is implicated in SBV pathogenesis (Barry *et al.*, 2014). Supporting this hypothesis, Varela *et al.*, 2013 demonstrated that SBV lacking NSs was attenuated *in vivo*.

Replication cycle

Very little is known about SBV life cycle and the data presented here is based on observations made on other bunyaviruses, mainly in the reference virus BUNV.

BUNV life cycle (Figure 5) starts when virus recognizes cellular receptors via their Gc/Gn surface glycoproteins (Borucki *et al.*, 2002 cited in Doceul *et al.*, 2013). Virus enters cells by endocytosis. pH decrease induces conformational changes in viral glycoproteins resulting in the exposure of a fusion peptide (Plassmeyer *et al.*, 2007 cited in Doceul *et al.*, 2013). This last allows viral and endosomal membranes to fuse, leading to the release of RNP in the cytoplasm. The transcription is initiated by a cap-snatching mechanism (Coupeau *et al.*, 2013), a process during which the L polymerase thanks to its endonuclease activity cleaves 10 to 18 nucleotides from the 5' end of a capped mature cellular mRNA and uses it as a primer to initiate viral transcription (Figure 6). Transcription will produce three kinds of RNA; anti-genomic RNAs that are used as templates to produce genomic RNAs, viral mRNAs that are translated by host ribosomes to form viral proteins and genomic RNAs that are incorporated as the genome in the new virions.

Viral proteins include Gc and Gn glycoproteins that form heterodimers in the endoplasmic reticulum and are transported to the Golgi where their glycosylation is completed (Walter *et al.*, 2011 cited in Doceul *et al.*, 2013). Viral proteins also include L and N viral proteins that are necessary for viral replication. The L polymerase produces new genomes copies associated with N and L proteins to form RNPs, these new genome RNPs accumulate in the Golgi and interact with the C-terminal domain of the glycoproteins (Hepojoki *et al.*, 2010 cited in Doceul *et al.*, 2013).

Viral particles are formed by budding of Golgi membrane depending on the Nsm protein whose precise role is unknown (Shi *et al.*, 2006). Mature virions then accumulate in vesicles and are transported to the plasma membrane where they are released by exocytosis. Once in the extracellular environment, virions can start a new viral cycle by infecting another cell.

It is important to keep in mind that a viral replication cycle may vary, depending on the cell type infected. Indeed, it has been observed during infection of mosquito cells with Bunyamwera virus that L and N proteins were sequestered in a large vacuole, leading to a decrease of viral production (Lopez-Montero *et al.*, 2011). This mechanism has not been observed in BHK-21 cells suggesting differences in the replication cycle of the virus among the infected cell strains.

In addition, it was observed in mammalian cells, that BUNV accumulates in the Golgi causing a swelling and a fragmentation of it and disrupting the secretory pathway (Salanueva *et al.*, 2003 cited in Lopez-Montero *et al.*, 2011). In mosquito cells infected with BUNV, there was no perturbation in the Golgi stacks. BUNV assembly was restricted to peripheral Golgi stacks and did not accumulate in it. Moreover, it has been reported that in mosquito cells infected with BUNV, virions are released by budding while in BHK-21 infected cells virions are released by extrusion, leading to cell death. These observations show again that the viral replication cycle may vary from one cell type to another.

To date, nothing has been published about ruminant Simbuviruses interaction with insect cells. Replication rate, interaction with cellular components, cytopathic effects, cells phenotypic modifications, RNA interference with replication and viral adaptation are still unknown for Simbuviruses in insect cells.

Origin

As SBV is a new virus that emerged in 2011, the question about its origin was raised promptly. As no indisputable evidence has been found about SBV origin, different hypotheses have been mounted. While Domingo *et al* (2010) suggested that viral emergence is a rare event and is more likely due to genetic changes, Jones *et al* (2005) suggested that viral emergence is frequent and most likely due to environmental changes.

According to Domingo *et al.*, viral emergences and re-emergences can be considered as episodes of virus adaptation to a new environment (Domingo *et al.*, 2010). Indeed, RNA viruses often present a high adaptability to changing environments due to their high mutations rates and large populations. However, the evolution of ongoing transmission into new host species is rare. Many of the RNA viruses that are considered as emergent are rather spillover infections (occurs when a reservoir population causes an epidemic in a novel host population) with no subsequent intra species transmission. It appears that arboviruses (arthropod born virus) are commonly the cause of spillover infections but they are rarely fully adapted to their new host transmission cycles. Despite their high aptitude to evolve, they cannot achieve their adaptation and so cannot be transmitted from one host to another. This paradox could be explained by the fact that adaptation to new hosts is often polygenic. Indeed, it implies for a virus to pass from a peak of fitness to another by traversing a period of low fitness where the virus is inadequately adapted to both host species (Holmes, 2006). This jump cannot be achieved in absence of high rates of reassortment and recombination because it requires gaining multiple advantageous mutations in a single replication cycle (Holmes, 2006). However, there are exceptions in which few mutations are sufficient to realize this jump of species as demonstrated by Anishchenko *et al* (2006) on Venezuelan equine encephalitis virus.

According to Jones *et al.*, 2005, 30 percent of all infectious diseases that emerged between 1990 and 2000 are due to arthropod-borne virus. The increase of travellers and trade exchanges as well as climate, ecological changes and the increase in livestock production should have contributed to the emergence of these diseases. Indeed, these last factors influence the probability that a new pathogenic virus enters in contact with a new serologically naïve host, leading to virus spreading. For example, an increase in the temperature in a geographic zone can result in modifications in the fauna and flora affecting the distribution of viral vectors (arthropods, birds...). According to Anishchenko *et al.* (2006),

ecological and immunological factors are the main determinant of successful viral emergence rather than genetic. For a viral emergence to occur, proximity between the recipient and the donor is required. The more contact between recipient and donors there are, the most likely pathogens will be exchanged. The number of contacts between donor and recipient is influenced by ecological factors as population density and weather.

In the case of SBV, it is hard to attribute its emergence to genetic or environmental factors. It is probably a combination of both that led to SBV outcome in 2011. SBV belongs to the Bunyaviridae family that shows high frequency of reassortments and as all RNA viruses is subjected to mutations. Therefore it is reasonable to assume that genetic factors are the starting point for SBV outcome and that environmental and immunological factors contributed to its spreading.

However it is hard to know which kind of genetic factors had the biggest contribution to SBV emergence: reassortment, recombination or adaptative mutations?

→ Mutations are universal and affect all viruses. They have an unpredictable effect that is reinforced by the fact that viral proteins are multi-functional. Mutations can affect one or more interactions between viral and host components (Lazaro *et al.*, 2003 cited in Domingo *et al.*, 2010). RNA viruses are more susceptible to accumulate mutations than DNA viruses. Especially, RNA/DNA viruses that have RNA as replication intermediate shows high level of mutations (10^{-3} to 10^{-5} miss incorporation per sites per year). Mutations occur during replication and are the result of misincorporation by the viral RdRp (RNA-dependant RNA polymerase) that does not possess a proof reading and no exonuclease 5'⇒3' activities while most of the DdDp (DNA dependant DNA polymerase) does. Therefore RdRp does not correct the mistakes during replication.

There are several types of mutations and their consequences vary according to their nature (Fields virology, 2007)

- Substitution includes transition and transversions. Transition is the replacement of a purine by another purine or the replacement of a pyrimidine by another pyrimidine. Transversion is the replacement of a purine by a pyrimidine or the opposite. These two kinds of mutations can be silent which means that they do not give rise to amino acid change, non-synonymous which means that they will give rise to a change in the amino acid sequence, or neutral which means that they do not affect fitness of the virus in a given environment. It is important to keep in mind that silent mutations even, if they do not change amino acid sequence can affect functions related to RNA structures as IRES, 3'UTR, miRNA binding....
- Insertion/deletion occurs preferentially in repeated sequences susceptible to misalignment and polymerase slippage. Their consequences depends on the number of nucleotides that are added/removed, if it's a multiple of 3 it adds/removes amino acids but if it's not a multiple of 3 it might shift the reading frame leading to truncated or aberrant proteins.

The consequences of these mutations are multiples; they can be neutral, lethal, beneficial, and they also may have different impact on the virus and on the host depending on their nature and their frequency.

According to Holmes *et al* (2006), even few changes in viral genetic sequence can influence virus fitness in new hosts, therein facilitating disease emergence. Theoretically, all SBV

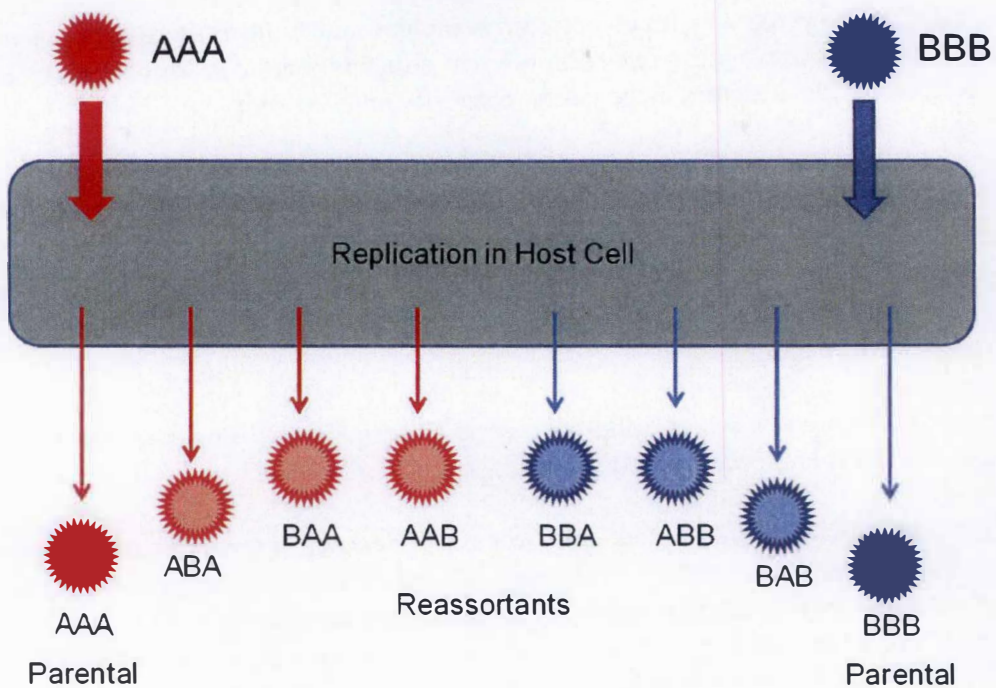


Figure 7: Potential reassortant produced by coinfection with two different tripartite bunyaviruses. Virus A (red) has three segments, S_A , M_A , L_A as virus B (blue) which possess S_B , M_B , L_B . When a permissive cell is infected in the same time with these two viruses, it may produce all the combinations that are shown behind the cell (the color intensity correspond to the frequency of the reassortant) (Briese *et al.*, 2014)

virions could present a mutation compared to its parental virus (due to mutations rate of 10^{-6} to 10^{-4} substitutions per nucleotide per cell infection (Sanjuan *et al.*, 2010) while SBV genome length is on average 11000 nucleotides). Mutations are therefore a plausible cause of SBV emergence.

→ Recombination frequency vary between viruses, according to Domingo *et al.*, 2010 this process is infrequent with negative single stranded RNA viruses. This is why this mechanism will not be developed in this work. However, it was described recently in an emerging bunyavirus in Thrombocytopenia syndrome virus (He CQ *et al.*, 2012). Even it is still being a plausible cause of SBV emergence, the genetic alignment performed did not provide any evidence of recombination within these Simbiviruses infecting ruminants.

→ Reassortment is restricted to segmented viruses. During this process, at least two viruses have to infect simultaneously the same cell. During packaging of the genome segments, they can be variously incorporated into the progeny viruses. This phenomenon leads to progeny viruses with a genome that is a mix of the parental segments as shown in Figure 7 (Briese *et al.*, 2013). The most common example of reassortment is influenza virus.

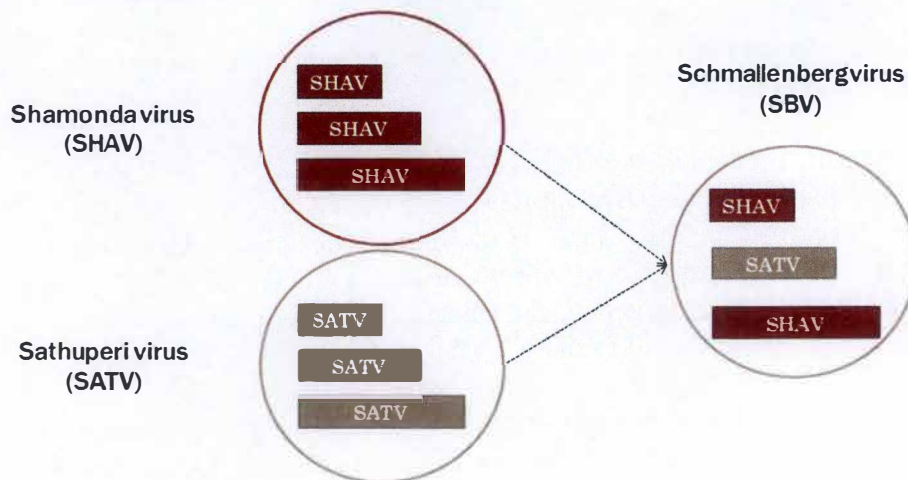
Through reassortment, influenza is subjected to antigenic shift which can confer an advantage in comparison of the ancient strains that are recognized by the immune system of the host. Reassortment can also introduce phenotypical changes, if a virus is highly infectious but not highly pathogenic and is mixed with another virus that is not highly pathogenic but highly infectious, it could results in three cases. First, it could result in progeny viruses that are low infective and low pathogenic, second in viruses that are the copy of the parents and third in viruses that are highly infectious and highly pathogenic. The last case was observed at each influenza pandemics observed in the common population (1918; H1N1, 1954; H2N2; 1968 H3N2)

In the case of arboviruses that are transmitted by *Culicoides*, it is also important to consider that dual infections of *Culicoides*, which do not produce antibodies against viruses, provide a good reservoir of cells supporting reassortment of viruses. In addition, the seasonal nature of *Culicoides* and the fact that arboviruses need vectors to be transmitted ensure that different arboviruses present in the same geographic location are transmitted during the same period. Moreover, as *Culicoides* feed frequently (depending on the species and weather), it raises the probability of a dual infection to occur, thereby leading to reassortment. These facts supporting again the hypothesis that SBV is a reassortant virus and raise the hypothesis that SBV may have appear through a reassortment process in *Culicoides* cells.

The requirement of compatible viral components should restrict reassortment between related viruses. Indeed, it is important to keep in mind that reassortment requires biological compatibility between the mixed segments to produce viable progeny (Briese *et al.*, 2013). All segmented viruses cannot be submitted to reassortment. For example, the occurrence of a productive or an abortive replication in the infected cell depends on the capacity of viral and host cells components to interact functionally (Briese *et al.*, 2013). The processing of viral polyprotein, the assembly of viral particles, the modulation of immune system and the egress of virions depend on viral/host interactions.

It has been demonstrated by Irorgbu and Pringle (1981) on Bunyamwera viruses and by Rodriguez *et al.*, 1998 on Hantavirus that reassortment is favoured between closely related viruses due to the restriction imposed by the viral components. Indeed, viral components should be compatible, therefore limiting the reassortment between very distant viruses.

Yanase hypothesis



Goller hypothesis

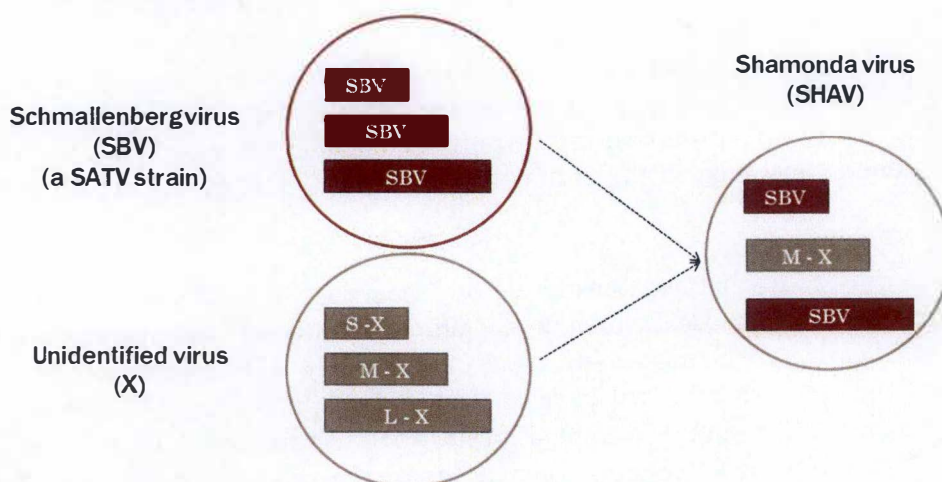


Figure 8: Schmallenberg virus origin hypotheses. The top picture represents Yanase hypothesis about SBV origin. The back picture represents Goller hypothesis about SBV origin

In addition, co-infection is restricted by precedent infections which leads to super-infection resistance. This phenomenon limits viral co-infection in a short period of time, just before the super-infection resistance takes place.

The compatibility between mixed viruses and the superinfection resistance are two constraints that limit reassortment between viruses that would not result in a genetic shift and support reassortment between viruses that are more likely to produce novel genetic traits (Briese *et al.*, 2013).

While the geographical origin and history of SBV remains unknown, two studies investigated on SBV origin by genomic analyses and reached different conclusion (Figure 8). The first one from Yanase *et al* 2012 suggested that SBV is a reassortant virus produced by a mix of the genome of Sathuperi and Shamonda, two other Simbuviruses. According to them, SBV arise from the S and L segment from SHAV and the M segment from Sathuperi. The second study from Goller *et al.* suggested that SBV could be the ancestor of Shamonda virus. During a reassortment process, SBV might have given its short and long segments to form Shamonda with the M segment of an unknown virus. However, sequence assignments of parent and reassortant may be ambiguous especially when the parents could be reassortant themselves (Briese *et al.*, 2013), this complicating the study of virus origin. Although it is not possible to choose between these two hypotheses for the moment, they appear to be plausible since reassortment process have been described for other Simbuviruses (Yanase *et al.*, 2010 cited in Doceul *et al.*, 2013) and bunyaviruses are all considered as reassortant (Briese *et al.*, 2013). Indeed, there are several examples of bunyaviruses that have been recognized as reassortants. The study of the orthobunyavirus and bunyavirus reassortants demonstrated that most of them possess the L and S segment derived from one virus and the M segment from another virus. This could be due to the fact that the interaction between polymerase, nucleocapsid, and RNAs that implies a closer match than what glycoproteins does. The explanation of these observations probably results in the way of packaging segments. However, the mechanisms of segments segregation into progeny virions remains less studied. The random packaging strategy appears to be less likely due to its hazardous efficiency and the sequence specific segregation more plausible. Kissing loops which are specific RNA interaction that have been showed to be implied in segment segregation in influenza virus are a plausible track. However, it is too early to infer this mechanism studied in influenza to SBV.

Transmission

As explained before, SBV host range seems to be limited to ruminants and there is no evidence of horizontal transmission within the same species. However, vertical transmission has been observed in infected sheep and cow during their gestations. When SBV infects a serologically naïve pregnant female after the placentomes (unit exchange between the mother and the progeny) are formed and before the blood brain barrier is functional in the foetus, viruses can pass from the mother to its offspring and infect fetus CNS. This infection results in severe syndromes as described in the previous part and often leads to the death of the animal. Knowing that there is no horizontal transmission and that vertical transmission leads to the death of the offspring, it is impossible that SBV has persisted and spread rapidly (as it was the case in 2012) without a vector.

Viruses of the *Bunyaviridae* family might be transmitted by arthropod vectors, as mosquitoes, phlebotoms, culicoides, ticks and thrips (Doceul *et al.*, 2013). For SBV it appears that its vectors are *Culicoides*, particularly the *C. obsoletus* complex, *C. chiopterus*, *C. dewulfi*

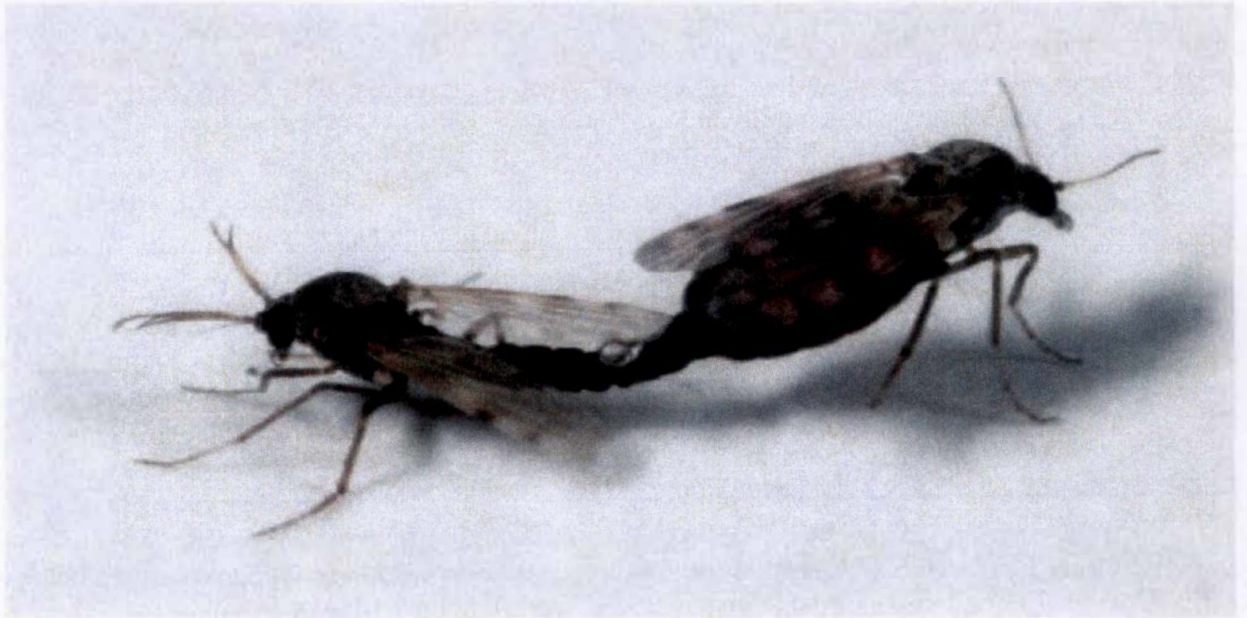


Figure 9: *Culicoides nubesculosus* coupling. Male is on the left and female on the right of the picture.
(Balenghien and Delécolle, 2009)

(De Regge *et al.*, 2012) and *C. sonorensis* (Veronesi *et al.*, 2013). Indeed, these species have been found positive for SBV genome in Belgium, Denmark, Netherlands, Norway, Poland, Sweden (De Regge *et al.*, 2012, Rasmussen *et al.*, 2012, Elbers *et al.* 2013, Doceul *et al.*, 2013). A recent study also showed that mosquitoes are not implied in SBV transmission, indeed, 50.000 mosquitoes trapped in 2011 in Germany were negative for SBV test in RT-RT quantitative PCR (Wernike *et al.*, 2014). However, it does not mean that *Culicoides* are the only vectors for SBV, even if mosquitoes seem not to be vectors for SBV, no studies investigated the ability of other arthropods to be vectors of SBV as ticks, thrips, black flies, phlebotoms.

As SBV re-emerged after winter 2011, (Claine *et al.*, 2012) it appears that it overwinters. However, it is not known, how SBV persists during this cold season. It may be in vector population surviving inside building and containing a latent virus, or in another reservoir. A study of Larska *et al.*, 2013 suggest that SBV could be transovarially transmitted to *Culicoides* offspring and therefore if the larvae overwinter, the virus do too. Indeed, they found two *C. punctatus* nulliparous females (females that have never been gestant and that never had any blood meal) that were positive for SBV. However, in the study of De Regge *et al.*, (2012) they examined 1359 nulliparous *Culicoides* females for SBV and they were all found to be negative. Further studies are needed to put the light on the transovarial transmission of SBV and on its putative reservoir.

Culicoides

Now that we are more familiar with SBV and its related viruses, take a look at its vector, the second main player in this work.

Generalities

Culicoides are small bugs of an average of 1mm in length that are classified as following:

- Embranchment : Arthropods
- Class : Insects
- Order : Diptera
- Family : Ceratopogonidae

There are more than 1400 species that have been identified, of which 96% are obligate blood suckers (Mellor *et al.*, 2000). *Culicoides* colonized nearly every land masses (except New Zealand, Hawaï, Iceland, Patagonia and Polar Regions) (Mellor *et al.*, 2000). Females and males are morphologically different (Figure 9). Their life cycle consists of egg, four larval stages, a pupae and the adult (Carpenter *et al.*, 2013). Eggs are not resistant to drying and hatch between two and seven days (Mellor *et al.* 2000). The duration of the larval stages depends on temperature and the species but may vary between five days and several weeks (Meiswinkel R. 1989 cited in Mellor *et al.*, 2000). The pupal stage is brief, generally from two or three days depending also from the species and the temperature. Adults are crepuscular for most of them and they live between 10 days and two months according to the species (Walzer, 2009). *Culicoides* can flight actively from hundred meters to a maximum of two or three km (Lillie *et al.*, 1981 cited in Mellor *et al.*, 2000), however, *Culicoides* can be dispersed passively by the wind over hundreds kilometers (Hayashi *et al.*, 1979 cited in Mellor *et al.*, 2000). The females are hematophagous and most of them are anautogenous which means that they need a protein intake to produce eggs, while the males are saprophagous which means

that they eat organic material in decomposition. So, due to their eating habits, only the female can transmit pathogens as viruses to vertebrates by biting.

Implication in arboviruses transmission

Although *Culicoides* can transmit several pathogens as protozoa and filarial worms to vertebrates (Mellor *et al.*, 2000) we will focus on their ability to transmit arboviruses as SBV. More than 50 arboviruses have been isolated from *Culicoides* and belong to the Bunyaviridae family, the Reoviridae and the Rhabdoviridae family (Meiswinkel *et al.*, 1994 cited in Mellor *et al.*, 2000).

To avoid any misunderstanding, it is necessary to define what an arbovirus is: "*A virus which can infect hematophagous arthropods, when these last ingest infected vertebral blood. Arbovirus multiplies in the arthropod's tissues and is transmitted by bite to other susceptible vertebrates*" (Mellor 2000). Therefore, any species of blood-sucking arthropods can be considered to have the possibility to transmit arboviruses, as long as they amplify the virus in their tissues. However, only five groups can transmit arboviruses: mosquitoes, biting midges (*Culicoides spp*), ticks (Ixodida), sandflies (Phlebotominae) and blackflies (Simuliidae) (Mellor, 2000). Other hematophagous arthropods as tsetse fly, horse fly, flea, are not considered as biological vectors of arboviruses but as mechanical vectors of other viruses (Mellor, 2000).

Competence and vectorial capacity

To characterize the ability of an arthropod to transmit a virus, two different concepts are used, the competence and the vectorial capacity.

Competence is defined as the intrinsic ability of a vector to support viral infection, replication and/or dissemination (Mullens *et al.*, 2004). Competence is influenced by extrinsic and intrinsic factors. Extrinsic factors are for example vector and virus density and climatic conditions as temperature. Intrinsic factors are genetic traits that have been inherited and that influence the ability of the vector to be infected by the virus after ingestion of viraemic blood and its ability to transmit the virus to hosts.

It is not possible to calculate precisely the vectorial competence since it is influenced by too many factors. However it can be estimated in laboratory with experimental oral infections of the vector. After the extrinsic incubation period (the time needed for viral dissemination and replication in the vector), the number of positive vectors is expressed in percentage. (<http://books.openedition.org/irdeditions/2705?lang=fr>, consulted on 25/09/2014)

Vectorial capacity can be defined as the ability of the vector population to transmit a pathogen, and is described using the following formula (Mullens *et al.*, 2004):

$$C = ma^2Vp^n / -\log_e p$$

- C: vectorial capacity
- m: bites per host per day
- a: host preference/ length of time between blood meals
- V: vector competence

- p: daily survival probability of the vector
- n: extrinsic incubation period

So, vectorial capacity and vector competence are very different concepts. The first one is subjected to environmental changes and the second is not. Indeed, adult midge competence is genetically determined (Craig et Hickey, 1967) but environmentally influenced (Tabachnick, 1996 cited in Mullens *et al.*, 2004). Similarly the oral susceptibility which is under the control of a single gene with a dominant allele for resistance but environmentally influenced (Jones and Forster, 1974 in Mellor, 2000). The temperature is the parameter that has the biggest influence on arbovirus transmission by *Culicoides*. Indeed, high temperature increases the number of blood meal taken by *Culicoides* and raises the virogenesis. On the other hands, higher temperature decreases the lifespan of the vector leading to less transmission. While, for lower temperature, the opposite occurs, vectors live longer but virogenesis is decreased. In addition, there are more subtle effects of temperature on arbovirus transmission: the ability of the vector to modulate viral replication and the threshold to overcome the barriers of replication and dissemination in the vector are influenced by the temperature (Mellor, 2000).

Arbovirus life cycle

The general life cycle of an arbovirus in its arthropod vector is the following:

- First, an infected arthropod female bites a susceptible mammal host and in the same time injects viral particles.
- Second, viruses amplify in the hosts until a certain minimal threshold.
- Then, a naïve female arthropod bites the viraemic host and ingests blood containing viral particles.
- As the meal is blood, it is directed to the hind part of the midgut by the contraction of a sphincter muscle.
- In the midgut, viruses attach to gut cells and replicate at their level. Then, virions are released at the basolateral pole in the haemocoel. Viruses disseminate through it and infect secondary organs as salivary glands that will allow oral transmission or reproductive organs that will allow venereal and transovarial transmission.
- When this infected vector bite a host, the life cycle starts again.

The duration, the tissues infected, the transmission rate, the numbers of infected insects, the titer of the virus produced depend on the virus, the arthropod host and the environmental factors (Mellor, 2000). None of these features have been characterized for SBV yet.

However, there are several barriers that may interact with the competence of a vector. The movement of a virus through arthropod body can be divided into three phases (Hardy, 1988 cited in Mellor 2000).

- Infection and multiplication in mesenteral cells followed by escape of progeny viruses in the hemocoel.
- Dissemination through hemacoel with or without secondary amplification in the cells of organs bathed in haemolymph.
- Infection of the salivary glands and subsequent oral transmission, or infection of the reproductive organs and venereal/transovarial transmission.

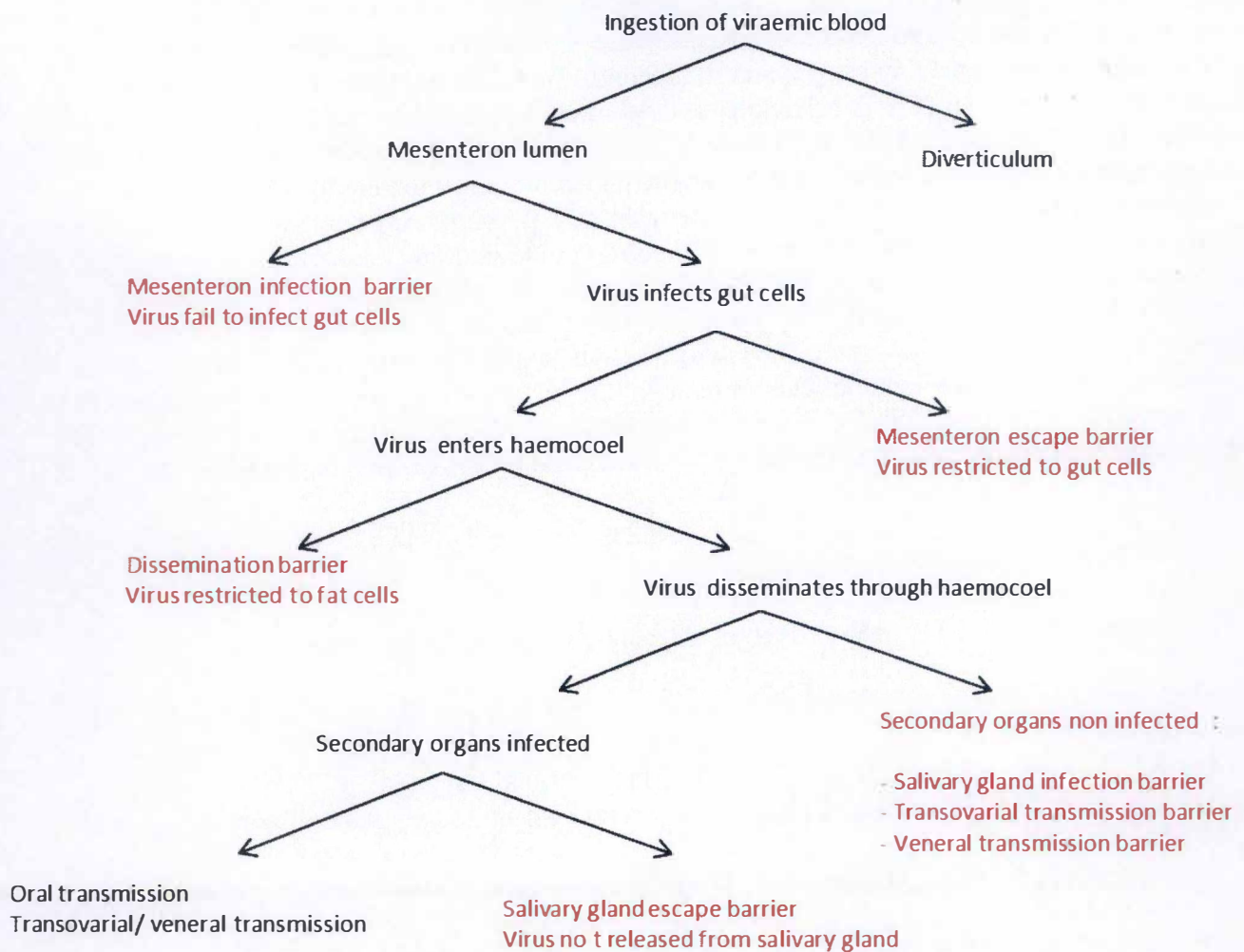


Figure 10: Possible ways taken by viruses in insect vectors and physiologic barriers on that ways. After the ingestion of viraemic blood by the vector, blood can be directed in the diverticulum (part of the gut) or in the mesenteron (middle part of the gut). There, viruses can be blocked by mesenteron infection barrier or uninfected gut cells or pass through the gut (leaky gut). Viruses enter hemocoel at the basal pole of gut cells or are blocked by mesenteron escape barrier. For viruses in the haemocoel, they can be blocked by the dissemination barrier or disseminate through it. After dissemination and amplification in the hemocoel, viruses can infect secondary organs or not (if there are salivary gland infection barrier or transovarial/veneral transmission barrier). If secondary organs are infected, viruses are orally/transovarially or venerally transmitted, but viruses may not be released from salivary gland if there is a salivary gland escape barrier (Modified from Mellor, 2000).

On that way, there are several barriers (Figure 10) that may influence vector competence (explained below). The presence or absence of these barriers seems to be hereditary traits (Mellor, 2000).

Barriers to viral competence

First, the mesenteron infection barrier (MIB), mesenteron escape barrier (MEB), second the dissemination barrier (DB) and third, the salivary gland infection barrier.

The mesenteron infection barrier was the first to be discovered. In 1933, Storey demonstrated that if the integrity of the mesenteron of *Cicadulina mbila* was disrupted with a needle, the insect that were not transmitters of maize-streak virus before, become transmitters after the manipulation. This suggested to him that the mesenteron is a barrier to viral transmission. The characteristics that render the mesenteron a barrier are multiple. It had been advanced that the mesenteron plays a role in the inactivation of the virus by diversion of the blood-meal in the diverticula, by secretion of digestive enzyme, absence or reduced number of virus-recognized receptors on the luminal pole of the mid-gut cells and abortive replication of the virus in the midgut cells (Hardy, 1988 cited in Mellor, 2000).

Studies on mosquitoes *Cx pipiens* oral resistance to infection by WEEV (western equine encephalomyelitis) lead to the conclusion that MIB could interfere with the attachment, penetration or uncoating of virus but not with the inability of the virus to replicate in the cells of the mesenteron (Hardy, 1988). Indeed, when Deae-Dextran is added to viraemic blood to reduce the electrostatic repulsion between anionic viral envelope and the luminal surfaces of the mesenterons cells, WEEV replicated in the mesenterons cells (Houk *et al.*, 1986 in Mellor *et al* 2000), likewise, when WEEV was inoculated parenterally, WEEV replicated in the mesenteron cells. Similar studies were performed on *C.variipennis*. Jennings and Mellor (1987) showed that some individuals were refractory to oral infection with BTV but all individuals could be infected parenterally, therefore supporting the hypothesis of a MIB. It was also demonstrated that this MIB is under the control of a single gene that carry a dominant allele for resistance to the infection (Jones and Forster, 1974 in Mellor, 2000).

Once viruses infect mesenteron cells, they can be blocked in their progression by a second barrier, the mesenteron escape barrier (MEB). Indeed, insects can be infected but unable to transmit the virus by biting. Jennings and Mellor (1987) showed that *C. variipennis* infected with BTV that present a titer $<2.5 \log_{10}$ TCID₅₀ were unable to transmit the virus while insects with a titer of BTV $>2.7 \log_{10}$ TCID₅₀ were transmitters of the virus. After dissection of the mesenteron of these insects, they showed that in the non-transmitters, viruses were restricted to the cells of the mesenteron and have failed to spread into the haemocoel. These insects present a MEB and although persistently infected, they were unable to transmit the virus due to the MEB that prevents the virus to pass from the mesenteron to the haemocoel.

The next barrier on the way of viruses in insect is the dissemination barrier (DB). It has been observed that some insects without a MIB or a MEB showed restricted infection to abdominal fat body and mesenteron cells. Knowing that fat body cells play a role in immunity (Rees *et al*, 1997 cited in Mellor 2000) it suggests they limit viral dissemination through haemocoel.

When viruses escape the mesenteron and fail to multiply in tissues bathed by the haemolymph, there is a salivary gland infection barrier (SGIB). However, mosquitoes exhibiting SGIB decrease over the course of infection suggesting that a threshold of virus is necessary in the haemolymph before the salivary gland can be infected.

The last barrier is the salivary gland escape barrier (SGEB). Its basis is poorly understood but there are several hypotheses. First, not enough viruses are produced by the salivary gland to infect a vertebrate. Second, with the time, the titer of viruses in the salivary glands is modulated to low levels. Third, the kind of cells that are infected in the salivary glands may have an important role. Indeed, Takahashi (1982) demonstrated that lateral acinar cells have to be infected to allow an efficient transmission.

Implication of *Culicoides* as vector of SBV

The rapid expansion of SBV and the fact that related viruses as Akabane are spread by arthropods led to the hypothesis that SBV could be spread by the same vectors. Therefore, it was reasonable to study *Culicoides* as potential transmitters of SBV. The first study is the one of Rasmussen (2012). In October 2011, they captured *Culicoides* in Denmark near to the border with Germany where first cases of SBV were discovered. They used the entire body of the *Culicoides*, they analyzed the pools for the S and L segment of SBV. Two of their 22 tested pools were positive and consists in *C. obsoletus* group.

Another study of De Regge *et al* (2012) supported this previous study. Indeed, they caught *Culicoides* near farms where SBV presence was confirmed. Midges were morphologically identified and pools of maximum 25 heads of parous females were prepared. These pools were analyzed by RT-RT qPCR using Agpath-ID One Step RT-PCR kit for the S segment of SBV. Among the 134 pools collected in Antwerpen, 5 were found positive and among the 44 pools collected in Liège, 7 were found positive. In these pools, there was *C. obsoletus* complex, *C. obsoletus* s.s., *C. dewulfi*, *C. chiopterus* and *C. pulicaris* (De Regge *et al.*, 2012). To confirm the positive status of the pools, a RT-RT qPCR was performed on the L segment and only *C. pulicaris* was not found positive for both segments. These results suggest that *C. obsoletus* complex, *C. obsoletus* s.s., *C. dewulfi* and *C. chiopterus* play a role in the transmission and spreading of SBV. The fact that they only analyzed heads of *Culicoides* straightened this hypothesis because it suggests that *Culicoides* are positive due to an amplification of SBV in the salivary gland and not due to the ingestion of a positive blood meal.

Other studies shown that *C. sonorensis* is a competent vector for SBV (Veronesi *et al.*, 2013) as well as *C. scoticus* (Elbers *et al.*, 2013).

After identifying the putative vectors of SBV, researchers studied how *Culicoides* led to the vast and rapid expansion of SBV. This has been studied by Sedda and Rogers (2013), a mathematical model based on BTV-8 outbreak has been modified with SBV outbreak parameters to understand how SBV spread through Europe.

Due to their small size, *Culicoides* can be transported passively by the wind. However, this is subjected to certain conditions of temperature, precipitation, humidity and wind speed (Burgin *et al.*, 2013 cited in Sedda and Rogers, 2013). Taking in account these parameters and the entomological parameters, it is possible to know the origin of an epidemic and to simulate it. For BTV-8, it has been proposed that it has spread by wind-borne midges since it was the only hypothesis that explained the direction and the extent of BTV in 2006 in Europe. After, the influence of the wind on BTV outbreak was confirmed (Burgin *et al.*, 2013 cited in Sedda and Rogers, 2013).

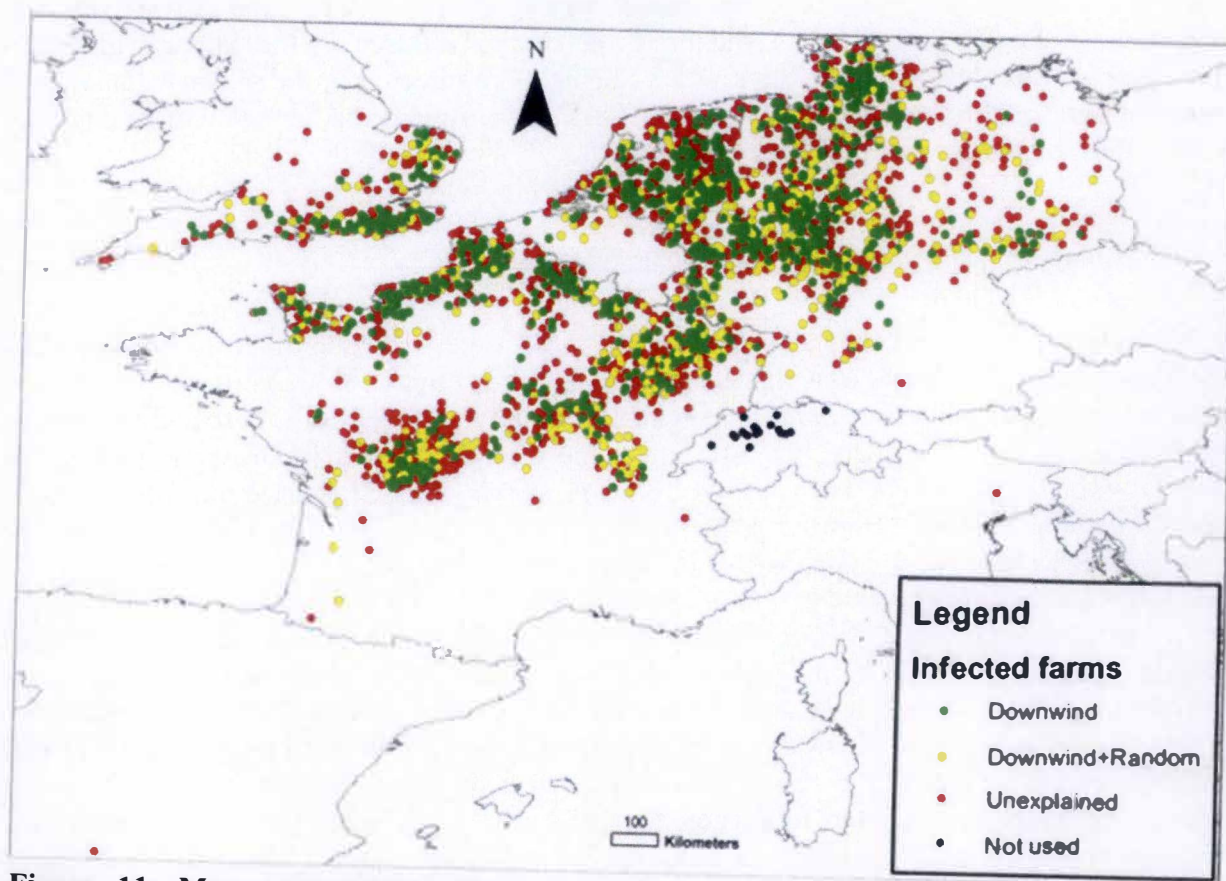


Figure 11: Map representing European farms positive for SBV infection between December 2011 and May 2012 according to the type of midge movements at the origin of the infection. In red (30%) are the infected farms whose infection is unexplained by the mathematical model, in green (43.4%) the farms that have been infected by downwind movement of midges, in yellow (26.6%), the farms that have been infected by the combination of downwind and random movements of midges and in black the farms that were not used in the model (Seda and Rogers, 2013).

It was supposed by Sedda and Rogers (2013) that SBV spread on the same way. The mathematical model developed here for SBV is based on the data of the SBV cases on farms reported by the OIE (World Organisation for Animal Health) from December 2011 to May 2012. On the 3487 affected farms, most of them were infected by midges arriving by downwind movement (43.4%), or a mix of random movements and downwind movement (26.6%) and 30% of the cases are unexplained by the model (Figure 11). The absence of farms infected by upwind movements (meaning by active flight of the midges in reaction of the odor of cattle) may be due to SBV hidden infected farms. Indeed, because the symptoms are not accurate in adults, some infected farms may be absent from the data on which the model is based. Therefore, the very long distances between the infected farms that are taken in account in the mathematical model render them impossible to have been reached by a *Culicoides* in active flight. The 30% of infected farms that are unexplained by the model may be due to approximate date of infection, approximate geo-localisation of the farm, unreported infections, other insects than *Culicoides* which are transmitters or an error in the model. However, the hypothesis that the wind influenced SBV outbreak in Europe appears highly plausible because of its rapid and extended diffusion.

Objectives

Schmallenberg virus was discovered in Germany in 2011 after serious damages that it caused in lamb and cattle offspring. Its discovery immediately gave rise to questions about its origin, transmission and pathogenicity. Its genome was rapidly sequenced and SBV was classified in the Bunyaviridae family, Orthobunyaviridae gender and the serogroup Simbu. Like other viruses of this family, SBV possesses a tri-segmented genome and is an arthropod borne virus (arbovirus). After sequences comparison, SBV has been related with Shamonda and Sathuperi viruses, two other Simbuviruses. Studies suggested that SBV is a reassortant virus issued from a simultaneous infection involving two Simbuviruses infecting ruminants, SATV and SHAV. Otherwise *Culicoides* has been showed to be competent vectors for SBV transmission (De Regge et al., 2012; Rasmussen et al., 2012). From these studies, it is obvious that SBV life cycle occurs in two different contexts since it alternates from mammal hosts to insect vectors. Therefore, SBV is submitted to different immune pressure in the different hosts and the vectors it infects. This alternate life cycle requires a high capacity of adaptation.

The first objective is to investigate on SBV behavior in both host species (vertebrates and insects) it infects. In that aim, *in vitro* studies will be performed in mammalian (BHK-21) and insect (KC) derived cell lines. Despite the fact that BHK-21 are not derived from natural host of SBV, they ensure multiplication of SBV *in vitro* and have been used in other studies on SBV. Viral amplification, the parameters influencing it and cytopathic effects will be studied in both cell contexts.

If differences are observed, the second objective will be to study the origins of these differential viral growth and differential CPE in both cells lines. From the literature, insect cells use several mechanisms to impair viral amplification. The first suggests that a mechanism of RNA interference limits viral growth in insect cells and the second reports that essential viral proteins are specifically trapped in insect cells thereby limiting viral replication. In addition, insect cells are described to be resistant to viral induced CPE as a consequence of a specific virus release mechanism that does not impair the cell membrane integrity. To investigate on the hypothesis of viral proteins trapping and on the different ways of viral egress, SBV interaction with cellular components will be characterized. To follow the course of SBV within the cells, antibodies raised against SBV will be produced. SBV N nucleoprotein is a good candidate as antibodies target due to its high level of expression in infected cells. A recombinant nucleoprotein will be developed and purified thanks to two tags that will be added at the N terminus of the protein. After purification, the recombinant protein will be analysed by mass spectrometry and 2D gel electrophoresis. After rabbit immunization with recombinant nucleoprotein, the polyclonal antibodies characteristics will be assessed by ELISA, western blot and immunofluorescence assays in mammalian and insect cells.

The third objective is to determine whether SBV will adapt to insect cells and raise its fitness through successive cellular passages. In that purpose, KC cells will be infected at long term and virus amplification ratio will be monitored through genomic amplification and infectious titre assays. SBV phenotypic adaptation in KC cells will be further analyzed by comparing partial genomic sequences at different cell culture passages with the original viral sequence. To this end, SBV S segment and the hyper-variable region of the M segment will be sequenced and analysed to study the accumulation of mutations in parallel of the viral adaptation.

This work on SBV interaction and adaptation with vector derived cell line will allow us to investigate on SBV origin. Indeed, SBV has been related to two others orthobunyaviruses, Shamonda and Sathuperi. The segmented structure of Bunyaviruses, combined with the accumulation of mutations gives this family a high evolutionary potential (Briese *et al.*, 2013; Tarlinton *et al.*, 2012). Therefore, it is reasonable to postulate that SBV could have emerged from a reassortment event involving SHAV and SATV. Knowing that SBV presents a two host alternate life cycle, reassortment should have occurred in one of these two hosts. Studies on viral growth kinetics in mammalian and insect cells demonstrated that SBV infection was acute in mammalian cells and viraemia was short in vertebrate hosts while infection was persistent in insect cells. Therefore, vectors provide a good reservoir of cells supporting reassortment of viruses rendering this host most likely implied in the origin of SBV than vertebrate host. In order to investigate on this hypothesis, co-infection experiments implying SATV and SHAV in KC cells are needed. However, this virus should present high rate of replication to allow study of progeny viruses. Therefore advances made in this work will be useful to understand how viruses replicate in hosts cells and interact with cellular components and which process rendered viruses adapted to KC cells.

This study will finally help us to understand how arboviruses emerged and how do they interact with the different hosts they infect. This would help us to predict which viruses could appear and how counteracting them to avoid important economic losses in the livestock industry caused by arbovirus emergence.

Materials and methods

Materials and methods

Cellular biology

Cell cultures

BHK-21 adherent cells propagated from baby hamster kidney were used. Despite the fact that these cells are not derived from natural host of SBV, they ensure multiplication of SBV *in vitro*. Indeed, this cell line is known to be defective in type I interferon (Truant *et al.*, 1977). BHK-21 were grown in complete GMEM that consists in GMEM (Glasgow Minimum Essential Medium) (Sigma) added with Tryptose broth phosphate (2.95gr/L), 1% penicillin-streptomycin (Lonza, 5000 U/ml for penicillin and 5000 µg/ml for streptomycin) and decompemented fetal bovine serum (FBS) (10%) at 37°C and 5% CO₂. Once cells reached confluence in the flask, GMEM is removed, PBS-EDTA (phosphate buffered saline - Ethylène Diamine Tetra Acétique) is added to chelate bivalent ions that are unfavorable to trypsinisation. PBS EDTA is removed and trypsin-EDTA is added for detaching cells by cutting adhesion proteins and chelating calcium ions that are required for cell adhesion by integrins. After 3 minutes, when cells started to detach, complete GMEM is added to dilute trypsin and inhibit its activity that would be damaging for cells at long exposure time. Cells are resuspended in complete GMEM, and fractions of them are seeded in new flasks containing fresh complete GMEM following a specific dilution from 1/2 to 1/10. Cells are incubated at 37°C and 5% CO₂.

KC cells from *Culicoides sonorensis* embryos that are SBV vectors were used to study SBV adaptations in KC cells. They were grown in Complete Schneider's medium that consists of Schneider medium (Sigma) added with 10% FBS (fetal bovine serum), Amphotericine B (1.25µg/ml) and Gentamycine sulfate (25µg/ml) at 25°C without CO₂. Once at confluence, complete worn Schneider was removed, cells were scraped and suspended in complete medium. Fractions of these cells were seeded in flasks containing fresh medium following dilutions from 1/2 to 1/10. Cells were incubated at 25°C without CO₂.

Infections

BHK-21 and KC cells were infected with a controlled multiplicity of infection (MOI) of SBV (isolate Bovine/BH80/11-4/Germany). The viral suspension was added to Schneider medium supplemented with one percent FBS for KC cells and EMEM (Eagle Minimum Essential Medium) (Lonza) plus 1% FBS and 1% P/S for BHK-21 cells. The inoculum was distributed in the wells containing KC or BHK-21 cells at 70% of confluence, incubated respectively at 25°C without CO₂ and at 37°C with 5% CO₂. After two hours, the cell cultures were washed three times with Schneider 1% FBS for KC cells and EMEM for BHK-21 cells. Complete Schneider medium for KC cells and complete GMEM for BHK cells was added for the indicated incubation period.

Viral growth kinetics

The kinetic starts when the inoculum is distributed in the wells, different times were chosen to sample supernatant and cellular pellets. For the supernatant, 300µl of the medium were

Table 1 Oligonucleotides used in sequencing SBV L and S segments after serial passage in KC cells

Name	Oligonucleotides sequence (5'→3')	Segment	Position on the segment	Purpose
FS1	CTCCACTATTAACACAGAAATATG	S	10-34	PCR
FS4	CACTATTAACACAGAAATATGTCAAGC	S	13-40	PCR
RevSNich	CTATCTTAACAGAAGCCTTGACG	S	818-796	PCR
RSSATV	ACAGAAGCCTTGACGTATAATG	S	810-789	PCR
F1 HVR	TACATGAACGACGTGGATTG	M (hvr1)	1225-1244	PCR
R1 HVR	GCTTACCTTGATGTGGCGT	M (hvr1)	2007-1988	PCR
Rev HVR1 N	TTTAAGTTGTAAATTTGACTGCG	M (hvr1)	2042-2020	PCR
F1HVRN	GGACAACTTTACAAACAAGTGC	M (hvr1)	1253-1274	PCR
newHVR2F	GTCATCCAGATCGCTTTGAG	M (hvr2)	1867-1886	PCR
f2hvr	GATTAGGTCGAGTCTTGCTTG	M (hvr2)	1933-1953	PCR
R2HVR	GAGGAATTATTGCTGGAAGG	M (hvr2)	2793-2774	PCR
R2HVRn1	CTAATACCATTGATACCTTGTC	M (hvr2)	2842-2819	PCR
SF	gtAGTAGTGAACCTCACTATTAAC	S	1-22	RT
SR	AGTAGTGTTCTCACTTATTAACATC	S	840-824	RT
HVRF	GGCAGCAAAGTATATCTTGACTG	M	1178-1201	RT
HVRR	TGTGCGACTCGATATAACTATTC	M	2865-2842	RT

sampled and stored at -80°C until titration assay. For the pellets, an average of 10^6 cells scratched with tips was collected, centrifuged at 300g for 8 minutes. After that, the supernatant was removed and the pellets were suspended in 200µl of complete Schneider medium. The samples were stored in a freezer at -80°C.

Titration on mammalian cell cultures

BHK-21 cells were used to titer viruses because they show an obvious cytopathic effect (CPE) after SBV infection. Approximately 5×10^4 cells were seeded in the wells of a 96 wells plate in 100µl GMEM and incubated overnight at 37°C with 5% CO₂. When the cells reach 80% confluence, serial dilutions from 10^{-1} to 10^{-12} of viral suspension were added for 2 hours of incubation. 100µl GMEM was added and cells were incubated for 3 days at 37°C with 5% CO₂. Three days later, 50µl of crystal violet (50ml absolute ethanol, 2g of crystal violet and 1L of distilled water) was added to each well for 30 minutes to stain the cells. After that, plates were washed and viral CPE were examined. The titer expressed as TCID₅₀/ml (Tissue culture infective dose 50%) was determined according to Reed and Muench method.

Molecular biology

RNA extraction

During growth kinetics, the medium of cells infected with viruses was collected and during long term infection in KC cells cellular suspension were collected. RNA was extracted from the supernatant using QiAmp viral RNA mini kit from Qiagen according to the user manual. To inactivate RNases and isolate viral RNA, the sample was first lysed under highly denaturing conditions, then buffer conditions are adjusted to provide binding of the RNA on the column, the contaminants were washed away thanks to two buffers and the purified RNA was eluted with RNase-free buffer. Purity and concentration of extracted RNA were determined using a Nanodrop 2000.

Reverse transcription

Reverse transcription was performed using Superscript III RT kit and specific primers for small and hyper variable region of the M segment (NEB, Table 1) according to the user manual. 1µl specific primers (100ng/µl), between 500 and 1000 ng of RNA and 1µl dNTP Mix (10mM each) were added to a nuclease-free microcentrifuge tube. Then, the mixture was heated at 65°C for 5 minutes. 4µl of 5x First-strand Buffer, 10 µmol dithiothreitol (DTT), 40 units RNase Inhibitor and 200 units of Superscript III RT were added to the previous mix. The tube was incubated 60 minutes at 55°C (for reverse transcription) and finally at 70°C for 15 minutes to stop the reaction.

Polymerase chain reaction (PCR)

Two PCR were performed, one for cDNA amplification of SBV small segment and SBV HVR (M segment) divided in two parts and one for positive bacteria (which contained insert of interest) screening.

Table 2 : Oligonucleotides used in qPCR one step SBV L segment

	Sequences (5'→3')	Position on the segment
SBV-L1-11F	TTGCCGTTTGATTTTGAAGTTGTG	379-402
SBV-L1-155R	TCAGGGATCGCAAATTAAAGAACC	523-500
SBV-L1-36FAM	FAM-TCATCCGTGCTGACCCCTCTGGAG-BHQ1	404-427

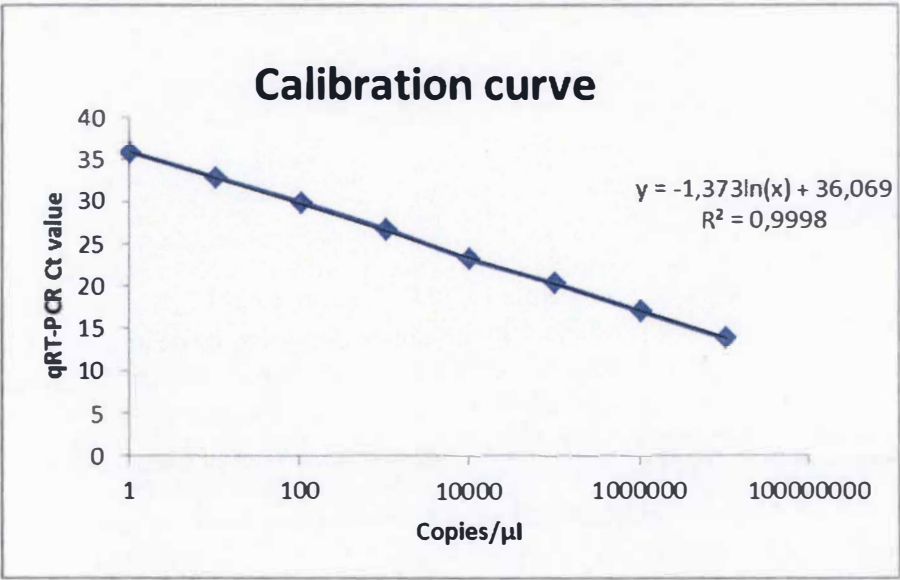


Figure 12 SBV calibration curve obtained from several dilution (from 10^{-11} to 10^{-18} g/μl) of SBV L segment CDNA obtained from the digestion in NotI of pGEM®-T Easy plasmid containing a fragment of SBV L segment (868 bp). A one step q-PCR was performed on this dilution series and the resultants Ct were used with the numbers of copies/μl (that were obtained as described in the material and method) to calculate the graph equation. R^2 is the coefficient of determination indicating how well the data fit with the equation of the curve

The PCR mix consists in 15µl/ml of GoTaq DNA polymerase (Promega, 5units/µl), 0.2µM of dNTP mix (England BioLabs Ambion, 10mM), Green GoTaq Reaction buffer 5x (Promega), 0.2µM of each screening primer (For cDNA amplification see Table 1 and for bacteria screening, Pu and RPU, Eurogentec: 5'-TGTAACACGACGGCCADTG-3' and 5'-CAGGAAACAGCTATGACCA-3', respectively). PCR was performed with 20µl of this PCR mix during 30 amplification cycles (30'' at 94 °C, 30'' at 55 °C and 1' at 72 °C).

Analysis of the resulting amplicons was carried out by 1.2% agarose gel electrophoresis. Stocks positive bacteria by PCR were sent for sequencing analysis (GATC). For negative cDNA amplicons, nested PCR was performed using a first PCR of 25 cycles (30'' at 94 °C, 30'' at 55 °C and 1'30'' at 72 °C) followed by a second PCR of 35 cycles with the same parameters.

Real time quantitative polymerase chain reaction

For virus growth kinetics, two kinds of real time quantitative polymerase chain reaction were used: the first one is a one-step RT-RT PCR targeting the L segment of SBV that consists in a reverse transcription and a quantitative PCR in the same tube. The second one-step PCR targets the S segment SBV and follows the same principle.

RT-RT one-step qPCR was performed in 10µl reactions containing 2µl template RNA, 5µl buffer 2X (AgPath-ID One-Step RT-PCR kit (Applied Biosystems, Ambion)), 0.8µl Mix SBV (containing forward primer SBV-L1-11F reverse primer SBV-L1-155R (Eurogentec) and TaqMan probe(AgPath-ID One-Step RT-PCR kit (Applied Biosystems, Ambion) SBV-L1-36 labelled with the fluorescent reporter dye FAM(6-carboxyfluorescein) at the 5' end and with the black hole quencher dye (BHQ1) at the 3' end) (Table 2), 0.4 µl of a mix containing reverse transcriptase enzyme and Taq polymerase (25x AgPath-ID One-Step RT-PCR kit (Applied Biosystems, Ambion)) and 1.8µl sterile distilled water in a 48-wells plate. First, the reverse transcription was performed at 45°C 10minutes, then the Taq polymerase was activated 10 minutes at 95°C, after what, a 3 step PCR was performed: denaturation at 95°C 15 seconds, hybridization at 56°C 20 seconds, elongation at 72°C 30seconds, during 40 cycles.

Calibration curve

A calibration curve was made to convert the Ct value obtained with q-RTPCR in a number of copies/µl. For that, SBV DNA obtained from the L1 segment of SBV (Coupeau *et al.*, 2013) cloned in a PGEM-T-easy vector digested with NotI restriction enzyme, were used. Dilutions from 10^{-8} to 10^{-15} g/µl were used and a one-step q-RTPCR on SBV L segment was performed on it. Once the corresponding Ct values were obtained, the following formula was used: $[(6.02 \cdot 10^{23}) \cdot x] / (868 \cdot 660) = \text{number of copies}/\mu\text{l}$ where x is the concentration in g/µl, to obtain the corresponding copies/µl. The CT values and the corresponding copies/µl were plotted and the equation corresponding to the calibration curve was obtained (Figure 12).

PCR products purification

To purify the PCR products of small and medium segment of SBV before insertion in pGEM-T Easy, the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel) was used. Its principle is

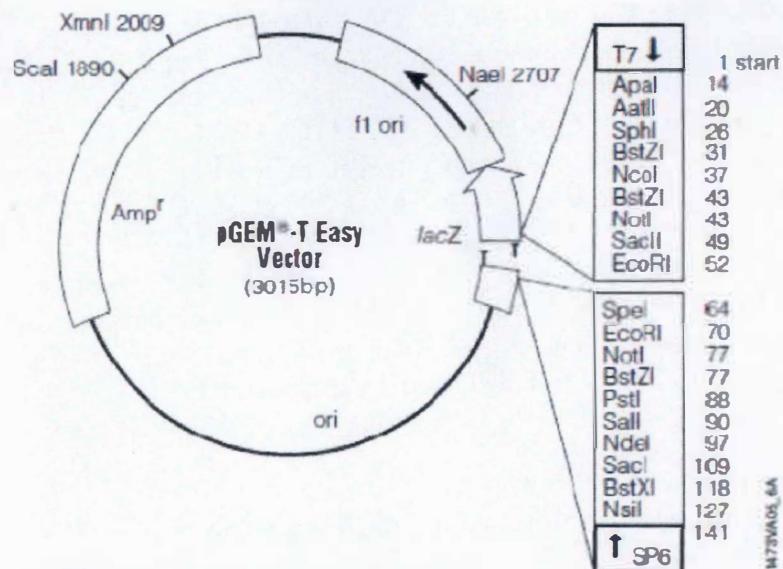


Figure 13 : pGEM-T Easy vector map. This vector was used to amplify SBV S segment and HVR region (M segment) in KC cells genetic experience.

based on rising DNA affinity for silice column by modification of salt concentration. A volume of solubilization solution (NTI) consisting of chaotropic salts is added to the DNA fragments in a ratio 2/1. The whole is centrifuged 1 minute at 11000g to fix DNA to the columns provided in the kit. Two washes with 650µl of NT3 buffer were made, followed by 2 minutes of centrifugation at 11000g for drying column and remove residual alcohol. To elute and collect DNA retained in the column, 18 µl of water was added before centrifugation 1 minute at 11000g.

PCR products ligation in pGEM-T Easy plasmid

pGEM-T Easy plasmid (Promega) (Figure 13) is a high copy plasmid made of 3015 base pairs used for direct cloning of PCR amplicons. It includes a bacterial origin of replication that allow the replication in electro-competent *E.coli*, a gene encoding a beta lactamase to select transformed bacteria on solid agar medium supplemented with ampicillin (100 micrograms/ml) and a lacZ operon encoding the subunit α of the β -galactosidase favor the α complementation in transformed bacteria in the presence of X-gal substrate (60mg /L) and IPTG (40 mg /L). Bacteria that are transformed with empty plasmid will form blue colonies while the bacteria that are transformed with recombinant plasmid will become white because of lacZ operon disruption by the inserted sequence.

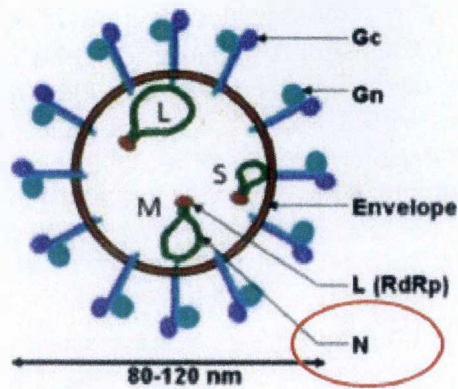
The pGEM-T Easy used in this manipulation has been previously linearized thanks to endonuclease clivage of EcoRV in the multiple cloning site (MCS) situated in the middle of *lacZ* operon and an asymmetric addition of thymidylate. Per samples, 5µl of ligation buffer, 1 µl of linearized pGEM-T Easy plasmid and 1 µl of ligase were mixed with 3 µl of purified amplicon. This solution was incubated overnight at 12°C.

Bacteria electroporation and culture

Electro-competent *Escherichia coli* (*E.coli*) TG1 are used to select pGEM-T Easy plasmids that have integrated amplicons of interest. To achieve this selection, each ligation product is incubated with *E.coli* following a ratio 1/50. This solution is placed in a 2mm vessel and electroporated (ECM 319 BTX) at 2500V for 5ms with an impedance of 25µF. This step permeabilized bacteria membrane and allow plasmids entrance.

Directly after electroporation, 150µl LB culture medium without antibiotic was added to the bacterias. After that, bacterias were grown overnight on solid LB medium supplemented with ampicillin (100 micrograms/L).

Colonies which are sufficiently isolated are stored by subculture on solid LB medium supplemented with ampicillin (100 micrograms/L) containing IPTG (40mg/L) and X-gal (60mg/L). In parallel, *E.coli* are suspended in a specific PCR mix to select colonies transformed with pGEM-T Easy plasmid containing the insert of interest.



His tag

S tag

Enterokinase

MHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLTDDDDKAMDPEFMSSQFIE
EDVPQRNAATFNPEVGYYVAFIGKYGQQLNFGVARVFFLNQKKAKMVLHKTAQPSVDLT
FGGVKFTVVNNHFPQYVSNPVPDNAITLHRMSGYLARWIADTCKANVLKLAEASAQIV
NPLAEVKGCTWADGYTMYLGFAPGAENFLTAFDFYPLVIENHRVLKDNMDVNFMKK
VLRQRYGTMTAEFWMTQKITEIKAAFNSVGQLAWAKSGFSPAARTFLQQFGINI

N ORF

Figure 14: SBV N nucleoprotein modified sequence. This sequence was used to produce recombinant SBV N nucleoprotein in baculovirus expression system.

Proteomic

Recombinant protein production

As the N protein of bunyaviruses is the most abundant viral antigen present in infected cells, it is the best target to develop antibodies in the aim of following SBV in infected cells (Lazutka *et al.*, 2014). SBV N nucleoprotein sequence was modified by addition of a His-tag and a S-tag (Figure 14) useful for the further purification steps.

To synthesize SBV N nucleoprotein, BioX company used bac-to-bac Baculovirus expression system.

The recombinant SBV N protein was introduced in a plasmid pFastBac, the recombinant plasmids were transfected in E.Coli DH10 Bac strain in which transposition occurred between a bacmid and the recombinant plasmid carrying SBV nucleoprotein gene. After isolation, amplification and purification of recombinant bacmid, insects cells (Sf9) were infected. The infection lead to the production of recombinant baculoviruses expressing the recombinant tagged SBV nucleoprotein. A large production of recombinant baculovirus was performed and SBV N nucleoprotein was produced and sampled in 10 litres of culture cell supernatant. After purification, the recombinant protein was loaded on a SDS PAGE gel to check its purity and its molecular weight.

Mass spectrometry analysis of SBV recombinant protein

To check the purity of the protein before rabbit immunization, mass spectrometry (MS) of recombinant SBV N protein was carried out. Because three bands were observed when the recombinant protein was loaded on a SDS PAGE, they should be studied separately.

10µg of SBV recombinant protein was mixed with loading blue5x (made of 10ml SDS 20%, 5ml β-Mercaptoethanol, 10ml Glycerol, 17.5mg bromophenol blue, 10 ml de spacer gel buffer (Tris-HCl 0,5M pH 6,8)) and heated for 5 minutes at 100°C. Sample was centrifuged few seconds on a minispin and the protein sample as the protein standard (SeeBlue Plus2 Prestained Standard protein kit) were loaded on a 12% Tris-Glycine gel (BIO-RAD mini protean TGX). Migration was performed at 150 volts, 400mA and 60W during 45 minutes. Proteins on the gel were stained using Silver stain.

Silver staining

First, proteins were fixed during 20 minutes in a solution made of 50% methanol, 5% acetic acid and 45% water. The gel was washed during 10 minutes in 50% methanol and 50% water and then incubated overnight in water to remove remaining acid. The gel was sensitized 1 minute in Sodium thiosulfate 0.02% solution. The gel was washed twice with water and incubated 20 minutes in silver nitrate solution 0.1%. The gel was again washed twice in water before incubation in a solution of formalin 0.04% and sodium carbonate 2% until coloration appear. The staining process was stopped using 5% acetic acid and stored in 1% acetic acid solution.

Digestion of samples

Before being submitted to mass spectrometry analysis, protein samples were reduced, alkylated and digested to form peptides. This process requires in three stages: elution, reduction and alkylation, digestion.

The first step was to cut the gel, it was cut in 8 pieces (Figure 17 b). Each lane fragments were divided in small pieces of 1mm^3 and isolated in Eppendorfs tubes containing $50\mu\text{l}$ of TD 50mM (digestion buffer NH_4HCO_3) and $100\mu\text{l}$ of water. Samples were eluted with water and incubated during 10 minutes under agitation at 900 rpm at room temperature. Supernatant was removed, $100\mu\text{l}$ of acetonitrile (ACN) was added, samples were incubated 10 minutes at RT under agitation at 900 rpm. To dry samples completely, they were placed in speed-vac during 5 minutes at 45°C .

The second step was to reduce and alkylate samples. To reduce disulfide bonds in protein samples, DTT 10mM (DiThioThreitol) was added, samples were incubated at 56°C under agitation at 600 rpm during 45 minutes. DTT was removed and iodo-acetamide 55mM was added to alkylate proteins (carbamidomethyl group of iodo-acetamide bind cysteine residues to avoid them to form disulphide bonds), samples were incubated 30 minutes in the dark. Liquid was removed and samples were washed with a solution of 50% ACN and 50% H_2O under agitation at 900 rpm during few minutes before removing liquid. $30\mu\text{l}$ ACN was added and samples were agitated at 900 rpm during 10 minutes, liquid was removed and $40\mu\text{l}$ TD 100 mM was added, samples were agitated at 900 rpm 5 minutes to dilute the remnant DTT and iodo-acetamide. After removing liquid $30\mu\text{l}$ ACN was added, samples were agitated at 900 rpm for 10 minutes, liquid was removed and samples were placed in speed vac for 5 minutes.

The third step was the digestion of the proteins by trypsin that hydrolysed amide function in peptides after Arginins and Lysins residues. Frozen TD 50mM was added to trypsin (Trypsin Gold 0,1 $\mu\text{g}/\mu\text{l}$, Mass Spectrometry Grade, Promega, WI, USA), samples were covered with trypsin and incubated on ice for 45 minutes. Trypsin excess was removed and TD 50mM was added, samples were incubated overnight at 37°C under agitation at 300rpm. Supernatant was harvested and transferred in a flask for analysis and stored at -20°C until use.

Mass spectrometry analysis

Once the recombinant SBV protein has been digested in peptides, samples are analyzed using mass spectrometry, more precisely by using ESI-MS/MS maXis Impact UHR-TOF (Bruker, Bremen, Germany) coupled with a nano-UPLC UltiMate 3000 (Thermo).

The first step of peptides mass spectrometry analysis is the decomplexification of the peptides mix using HPLC with a reversed phase column (C18). This last separates peptides according to their hydrophobicity. It is composed of a stationary phase to which peptides bind and a mobile phase (acetonitrile and water) which competes with peptides attached to the column. Hydrophilic peptides emerge first and hydrophobic peptides come out then.

Desorbed peptides are sent to the mass spectrometer via a capillary. Mass spectrometer is made of three parts: the source, the analyzer and the detector. As part of this work, an ESI-QUAD-TOF mass spectrometer was used. The source is ESI (electrospray ionization), the analyser is the Q-TOF which is actually a double analyzer that consists in a quadrupole and a time of flight separated by a collision cell. This assembly allows the sequencing of peptides and thus to check sample purity.

The first step in mass spectrometry is peptides ionization for transferring molecules from the liquid phase to the gaseous phase. Here, by using ESI, the peptides solution is dispersed in highly charged droplets by passing through a needle placed in an electric field. Indeed, the electric field will charge each peptide contained in the solvent, leading to repulsion charge, then the droplets moved in an evaporation chamber in which the solvent is evaporated. The combination of charge repulsion and evaporation of the solvent ensure that each droplet contains only one loaded peptide.

The second step of MS is to separate ions. Here the quadrupole (Q) selects a peptide based on its mass to charge ratio (m/z). Those corresponding to this ratio are selected among others and crossed the quadrupole in oscillating. The selected peptides are sent on the collision cell where a noble gas (azote) breaks peptides into peptidic fragments when collision occurs by a process called collision-induced dissociation (CID) where peptidic bonds are cleaved. Peptidic fragments were then moved into a second analyzer (TOF). This last separated peptidic fragments according to their m/z ratio and also according to their speed and time of flight to the detector.

The last step was the detection; results appeared as peaks, a peak corresponding to the m/z corresponding to an ion. To identify peptides, peak list was transfer in Mascot 2.4 that was used as search engine. Following parameters were used: enzyme specificity was set to trypsin, the maximum number of missed cleavages per peptide was set at two, carbamidomethylation, oxidation of methionine and Gln – pyro-Glu were allowed as variable modification, mass tolerance for monoisotopic peptide window was 10 ppm and MS/MS tolerance window was set to 0.05 Da. By comparing this peak lists with Schmallerberg database (containing Schmallerberg proteins and recombinant tagged N nucleoprotein), we were able to assess whether analysed peptides matched with Schmallerberg proteins or not. We have also used a complete database (UNIREF100) to determine if there were contaminants of other species in our analyzed peptides.

Dephosphorylation assay

To investigate on the phosphorylation level of the recombinant SBV N nucleoprotein, a dephosphorylation assay was performed using a recombinant phosphatase (Lambda Protein Phosphatase BioLabs). Samples were mixed according to the following table to reach a final volume of 50 μ l, and were incubated 30 minutes at 30°C.

	SBV nucleoprotein	NEBuffer 10X	MnCl ₂ 10mM	Phosphatase 400 000 unit/ml	Water
Lanel	10 μ g	5 μ l	5 μ l	/	38 μ l
Lane2	10 μ g	5 μ l	5 μ l	100 units	37.5 μ l
Lane3	10 μ g	5 μ l	5 μ l	200 units	36.75 μ l
Lane4	10 μ g	5 μ l	5 μ l	1000 units	35.5 μ l

After phosphatase treatment, samples were mixed with loading blue 5x (10ml SDS 20%, 5ml β -Mercaptoethanol, 10ml Glycerol, 17.5mg bromophenol blue, 10 ml de spacer gel buffer (Tris-HCl 0,5M pH 6,8)) and were heated 5 minutes at 100°C. Samples and molecular reference (SeeBlue® Plus2 Pre-stained Protein Standard) were loaded on a 15% acrylamide Tris-Glycine gel:

Stacking gel :

1,25 ml Tris 0.5M, pH 6.8, 0.4% SDS
0,5 ml Acrylamide 30% -Bisacrylamide
0.8%
2,75 ml water
5µl TEMED
0,5 ml APS 1%

Running gel :

2,5 ml Tris 1,5M pH8.8 0,4%SDS
4,95 ml Acrylamide 30%- Bisacrylamide
0.8%
2,05 ml Water
10µl TEMED
0,5 ml APS 1%

Migration was performed 1 hour at 200 volts and proteins were stained using Silver staining (as previously explained).

2D-gel assay

First dimension electrophoresis (isoelectric focusing)

In the first dimension, proteins are separated by using their isoelectric point which corresponds to the pH level at which protein do not carry any net electric charge. First dimension was performed on an immobilized pH gradient (IPG) strip (Immobiline Dry Strip pH 3-11 NL, 24cm, GE Healthcare).

First step was to re-hydrate the dry strip. This one was placed in plastic manifold with 450 µl of rehydration solution (DeStreak rehydration solution, Ge Healthcare) during 15 minutes at RT. It was covered with oil overnight to ensure that rehydration solution does not evaporate and concentrate. The next day, strip was placed face up on a ceramic manifold filled with oil. Sample was prepared by mixing 25µg of SBV N nucleoprotein (5µl) with the same volume of reduction buffer (7M urea, thiourea 2M, Chaps 2%, IPG 2% and DTT 130mM). Cupule system was used to charge sample on the strip. Migration occurred on IPGphor (Pharmacia Biotech) during 3 hours at 300 volts, 8 hours at 1000 volts and 3 hours at 8000 volts to finally reach 25 000 volts/hour. Strip was frozen at -20°C until utilization.

2 dimensional sodium-dodecyl sulfate-polyacrylamide gel electrophoresis

In the second dimension, proteins are separated according to their molecular weight thanks to SDS which confers a negative charge to all proteins. Before starting, a bind silane treatment was performed on one of the two glasses which are used for gel casting. Silane which is fixed on the glass plate will bind covalently the acrylamide in the gel and prevent the gel from moving when the spot Breakers take off proteins. Therefore, 2ml ethanol 96%, 50µl acetic acid 100%, 2.5µl bind silane and 450µl water were mixed. 2ml of this solution was spread on the glass plate which was then dried for 1 hour 15 minutes. After what glass was washed with water to remove silane excess and plates were completely dried before gal casting.

After that, a SDS PAGE gel 12.5% acrylamide of 24cm width and 1mm thickness was prepared by mixing 112.5ml Tris (1,5M pH8.8), 0.45g SDS, 187.5ml Acrylamide-Bysacrylamide 30/0.8%, 2.25 ml APS 10%, 148.5µl TEMED and 141ml water. Gel polymerization occurred overnight.

The next day, first dimension gel was incubated 15 minutes in DTT solution (100mg in 10ml equilibration buffer: Tris-Hcl (1.5M pH8.8) 6.7ml, Urea 72.07g, Glycerol 85% 69ml, SDS 4g,

H₂O to reach 200ml) and then 15 minutes in Iodoacetamide buffer (250mg Iodoacetamide in 10ml equilibration buffer). After that, first dimension was sealed above second dimension in electrophoresis buffer 10x (Tris 60,5g, Glycine 288g, SDS 2g, pH8.5) supplemented with agarose 0.5% and 200µl of Bromophenol blue 1%.

Gel staining

Krypton staining was chosen to stain proteins. Gel was incubated in fixation solution (40% ethanol, 10% acetic acid, 50% H₂O) for 30 minutes twice. After that, gel was washed 5 minutes in water and incubated in krypton solution (Krypton Protein Stain, Thermo Scientific) diluted 10 times in water for a minimum of two hours. Gel was decolorated during 30 minutes in a solution of 5% acetic acid before two successive washes in water for 15 minutes.

Gel imaging and spot picking

After coloration, gel was scanned with Thyphoon 9400 variable mode Imager. Using Decyder 2D program, positions of the spots to pick were defined. After that, the data were transferred to Ettan spot picker that picked up 38 spots in the gel.

Rabbit Immunization with SBV recombinant protein

All procedures involving experimental rabbits were performed under controlled laboratory conditions in strict accordance with the Belgian and European legislation. The immunization procedure was approved by the local welfare committee (agreement number: LA1900054, FUNDP 08/166). Two specific pathogen free Californian rabbits (confil company) aged of 12 weeks were immunized three times. Blood was sampled after each immunization.

At the beginning of the protocol, rabbits were immunized for the first time with 600 µl of SBV N nucleoprotein (corresponding to a dose of 3mg) mixed with Stimune adjuvant (Prionics). Rabbits were sheared on the back and solution was injected intra-dermally in 20 different sites. Four weeks post immunization, 5ml of blood was sampled from the major ear artery. Samples are called L295-1 for rabbit 295 and L296-1 for rabbit 296. The second immunization was performed the same day (week 4). 400µl of SBV N nucleoprotein (1mg/200µl) mixed with Stimune adjuvant was injected intradermally 20 times in the back area of both rabbits. At week 8, 5ml of blood was sampled for each rabbit. Samples are called L295-2 and L296-2. Third immunization was performed at week 9. 500µl of SBV N nucleoprotein mixed with Stimune adjuvant was intradermally injected 20 times in the back area of both rabbits.

All blood samples were taken in dry tube, placed at 37°C during 30 minutes to induce clotting. After that, the coagulate was centrifuged during 20 minutes at 400g at 20°C to separate serum from figurate elements and coagulation elements. Serum was frozen at -20°C until utilization.

Indirect ELISA assay

To assess whether rabbit serum contained antibodies rose against SBV nucleoprotein, and to check their specificity and their concentration, ELISA was performed. Wells of 96 wells

plates were coated using 1 µg of SBV nucleoprotein per wells. Nucleoprotein was diluted in coating buffer (pH 7.4, 8.5g NaCl, 1.4g Na₂HPO₄, 0.2g NaH₂PO₄, water to reach 1L) and 100 µl of the mix was added per well. Plate was incubated at 37°C for two hours. Wells were washed using 200µl of washing buffer per well (coating buffer added with 0.05% Tween 20) three times. To reduce non-specific Ig binding, 200µl of blocking buffer was added (washing buffer with 10% BSA), plate was incubated one hour at 37°C. Plates were washed three times using washing buffer. Rabbit sera were diluted in coating buffer and 100 µl of the adequate solution was added per wells, plate was incubated one hour at 37°C. Wells were washed with 200µl washing buffer three times. Secondary antibody (Sigma A0545 anti-rabbit IgG whole molecule) Peroxidase antibody produced in goat) was diluted in coating buffer (1:30000), 100µl of the solution was added per well, plate was incubated 30 minutes at 37°C. Wells were washed five times using washing buffer. To detect peroxidase activity, 100µl of TMB reagent (ID Vet Solution revelation) was added per well, after 5 minutes of colour development, 200µl of stopping buffer was added to stop the reaction. Absorbance at 450 nm was performed using automated spectrophotometer (Thermo Electron corporation Multiskan ex).

Western blot assay

To assess which proteins are recognized by primary antibodies produced in rabbit, western blot assays were performed by using infected and non-infected BHK, purified SBV N nucleoprotein, infected and non- infected KC cells.

Sample preparation

Cells samples were counted using Neubauer cell, centrifugated 8min at 1000rpm, washed with PBS and resuspended in the adequate volume of PBS to obtain the desired concentration. The adequate volume of cell suspension was mixed with 4µl sampling buffer (from a 5x solution made of 5ml of β- mercaptoethanol, 2g SDS, bromophenol blue, 5ml Upper Tris Buffer), PBS was added to reach 20µl of final volume. Samples were frozen at -80°C, heated 10 minutes at 100°C and frozen again at -80°C.

Electrophoresis and blotting on membranes

5ml of running gel was prepared through mixing 2.4ml H₂O, 1.25ml Lower Buffer (pH8.9, Tris 1.5M, 10%SDS, 3% EDTA), and 1.35ml Acrylamide/bisacrylamide 40% (37.5:1), 25µl APS 10% and 5µl TEMED. After polymerization, stacking gel was prepared through mixing 1.24ml H₂O, 500 µl Upper Buffer (Tris 0.5M, 10%SDS, 3% EDTA, pH 6.8), 270 µl Acrylamide/Bisacrylamide 40% (37.5:1), 10µl APS 10%, 5µl TEMED. After polymerization, gel was placed in electrophoretic tank with running buffer 1 x (from running buffer 10 x: 0.25M Tris, 10% SDS, 1.72% EDTA, 1.9M Glycine, pH 8.75). 20µl of samples were loaded per lane and 5µl of Page ruler Plus Prestained Protein Ladder (Thermo Scientific) was used as molecular reference. Electrophoresis was performed at 150 volts, 60 watts for 1 hour. Gel was washed in urea 4.5M during 45 minutes.

Proteins samples were passively transferred on membranes (Parablot nCP Nitrocellulose membrane, Macherey-nagel) using transfer buffer 1x (prepared 10x concentrated from transfer buffer pH 7.0 made of 100ml Tris 1M pH 7.0, 100 ml NaCl 5M, 7.45g EDTA in 1L

water) overnight. Membrane was saturated in TBST (0.2 M Tris pH 7.0, 1.5 M NaCl, 0.05% Tween 20, 1L water) added with 3% dehydrated milk.

Incubation with primary and secondary antibodies and revelation

Membranes was rinsed in TBST 1x and incubated in 15ml of primary antibodies (rabbit serum L295 and L296) diluted in TBST (1:100 for dilution) under agitation for 1 hour. Membrane was washed in TBST added with 1% dehydrated milk three times during 5 minutes to eliminate unbound antibodies. Membrane was rinsed with TBST and incubated under agitation 45 minutes with secondary antibody (Sigma A0545 Antibody IgG-whole molecule- Peroxidase Antibody produced in goats) diluted 1:80.000 in TBST. Membrane was washed three times during 5 minutes in TBST added with dehydrated milk 1%. Membrane was rinsed with TBST, and then covered with 750 µl of Femto revelation solution (Kit supersignal west femto maximum sensitivity substrate Thermo 34094). Revelation occurred in the dark during 2 minutes and signal was analysed by using a chemi-luminescence camera (Image Cant 350). Different exposure times were used from 10 to 250 seconds.

Immunofluorescence assay

BHK-21 and KC cells were seeded in 12 wells plates, on cover slips previously incubated with decompemented FBS at 37°C for three days. After they reached 70% of confluence, cells were infected with SBV for 2 hours in GMEM at 37°C and 5% CO₂ for BHK-21 and in Schneider 1% at 25°C without CO₂ for KC cells. BHK-21 cells were covered with agarose 0.35% for three days, incubated at their growth culture conditions. Agarose was removed and cells were rinsed three times with PBS and cells were fixed using 400µl per wells of paraformaldehyde (PAF) 4%. After 5 minutes at room temperature and 15 minutes at 4°C, PAF was removed and cells were washed three times with PBS. Cells were permeabilized using PBS added with NP40 0.01% for 10 minutes at 37°C. Cells were washed once with DPBS and once with PBS- FBS10% during 10 minutes.

Cells were incubated first by the primary antibody (rabbit serum L295 and L296) diluted in PBS-FBS 10% at 1:100 dilution for 1 hour at 37°C. After three washes of 10 minutes in PBS-FBS 10%, cells were incubated with secondary antibody (sigma, anti-IgG rabbit, whole molecule conjugated with Alexa 488) diluted at 1:1000 for 1 hour at 37°C. Cells were washed three times using PBS-FBS 10% during 10 minutes and once with PBS.

Cover slips were immersed in distilled water, remnant water was aspirated and cover slips were returned on 25 µl of Mounting medium (1ml of mounting medium Invitrogen molecular probes mixed with one tube of antifade reagent Invitrogen molecular probes) on glass lamels. The next day, cells were observed using epifluorescence microscope (Zeiss Axiovert40CFL), pictures were taken with AxioCam ICcl.

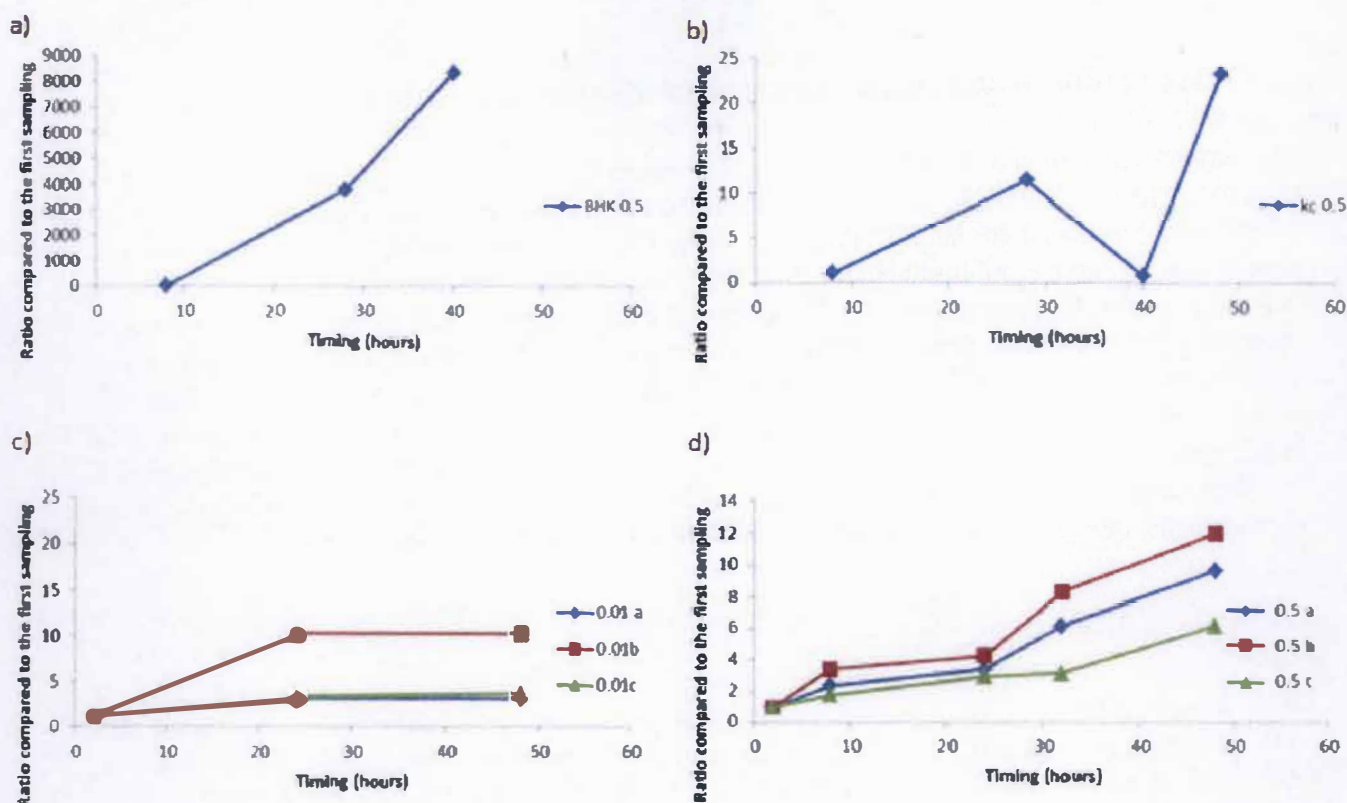


Figure 15: Graphs showing SBV amplification ratio over the time during growth kinetics in KC/BHK-21 cells. Kinetics were performed on 6 plates wells, cells were infected following a controlled MOI (see each graph own legend), RNA was extracted from supernatant as described in the material and method at different times. SBV numbers of copies were calculated thanks to the calibration curve and the ratio of amplification was calculated by dividing the number of copies / μ l at each sampling time on the number of copies/ μ l of the first sampling time.

a-b) SBV amplification ratio in BHK-21(a)/KC cells (b) cells infected with a MOI of 0.5

c-d) SBV amplification ratio in KC cells infected with an MOI of 0.01(e) / 0.5 (f) in triplicate

Results

Results

Interaction between Simbuviruses and *Culicoides* cells

Viral kinetics

This work aims at characterizing the interaction of SBV, SATV and SHAV with their vectors *Culicoides*. *Culicoides* are few studied and due to the recent emergence of SBV and the lack of zoonotic transmission of SBV, SATV and SHAV, the relationship between these Simbuviruses and their vector is poorly defined. Viral kinetics were established *in vitro* as a first approach to investigate on the interaction of Simbuviruses with *Culicoides*.

As SBV infects mammals and is transmitted by *Culicoides*, this virus must be able to replicate in very different cellular contexts. To assess the differences in SBV replication capacities in mammalian and *Culicoides* insect cells, viral growth kinetics were performed in KC and BHK-21 cells. Cells were infected with a controlled MOI of 0.5. Supernatant was collected at 8, 28, 40, and 48 hours post infection (P.I). The number of SBV copies/ μ l was calculated thanks to the calibration curve (Figure 12). The data were transformed in amplification ratios by dividing the viral concentration (expressed as genome copies/ μ l) at any sampling time by the viral concentration of the first sampling time. The ratios were reported to compare viral growth rates in mammalian (Figure 15a) and in insect (Figure 15b) cell cultures. For BHK-21 cells, the amplification ratio rose sharply over time to reach 8360 at 40 hours P.I. For KC cells, a peak (11.4) was observed at 28 hours P.I followed by a decrease (0.7) under the initial rate of viruses at 40 hours P.I after what the ratio increased again (23) at 48 hours P.I. Comparison of KC and BHK-21 curves showed that the amplification ratio was 363 times more pronounced in BHK-21 than in KC cells (8360 versus 23).

To assess if the number of viruses per cells during infection have an impact on the replication rate, and on the amplification kinetic, infections with a weak and a strong MOI were compared. For that, KC cells were infected with a controlled MOI of 0.01 and 0.5 in triplicates. A graph was generated that shows us for MOI 0.01 a and c samples (Figure 15c) an amplification ratio of 3 times 48 hours P.I while for b sample an amplification ratio of 10 times at 48h P.I was observed. The three curves had a similar appearance and showed a plateau between 24 and 48h P.I. For KC cells infected with MOI 0.5, we also observed that the repeatability was poor. Although, the curve appearance of SBV amplification ratio was similar between the triplicates with a low amplification until 24 hours post infection and a strongest amplification until 48hours post infection (Figure 15d). For 0.5 MOI, we observed a better amplification ratio than for a 0.01 MOI. Indeed, maximal amplification ratio was observed at 48 hours post infection and reached 9.6, 11.9 and 6.2 for a, b and c respectively.

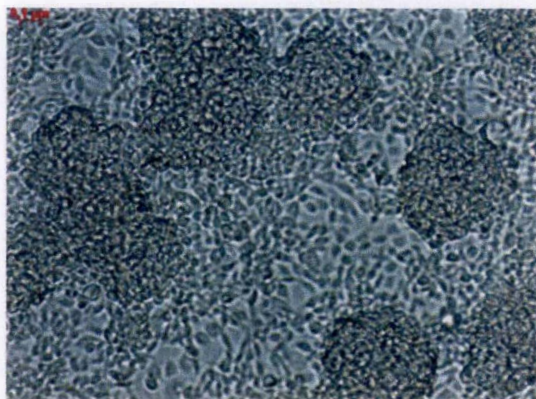
Cytopathic effects of SBV infection

Cytopathic effects (CPE) of SBV infection were investigated in KC and BHK-21 cells. Cells monolayers were monitored for the appearance of CPE every day during SBV infection. In BHK-21 CPE was obvious from two and three days post infection depending of the MOI. CPE were characterized by rounded cells in foci and refractile cells. These foci enlarged and formed large plaques in the cell monolayer with detached cells floating in the culture media.

KC cells

BHK-21 cells

Mock infected
cell culture



Infected cell
culture (72h pi)

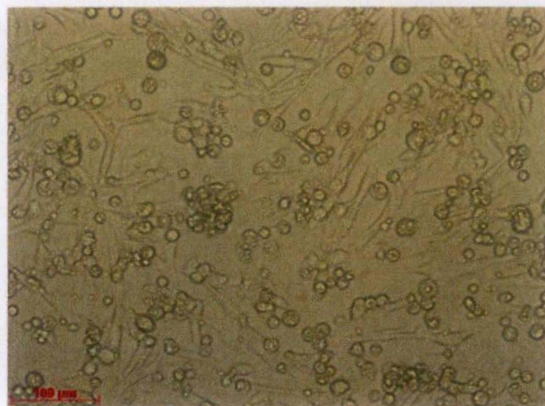
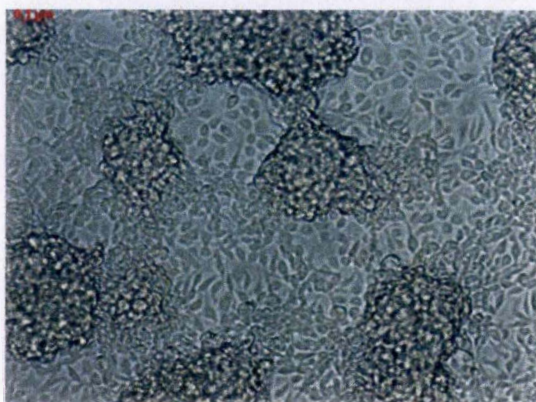
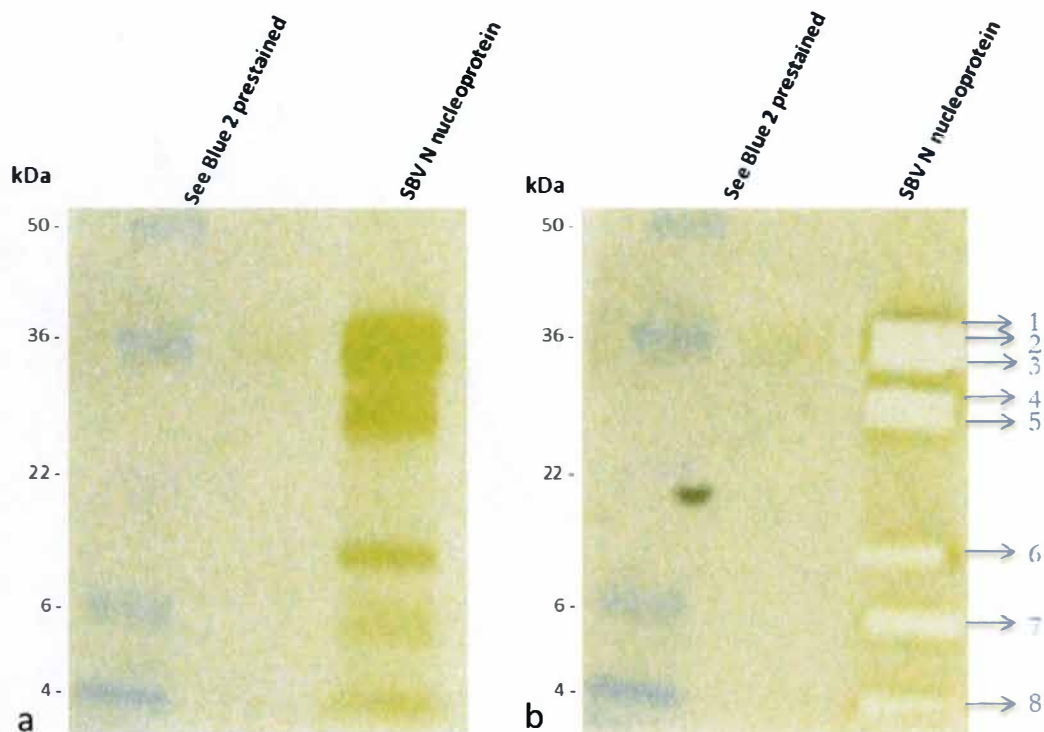


Figure 16: Cytopathic effect due to SBV infection in KC and BHK-21 cells. In the left panel are KC cells and in the right panel BHK-21 cells. Pictures of mock infected cell culture and infected cell culture were taken in phase contrast microscopy 96 hours post seeding and 72hours post infection for mock infected cells and infected cells, respectively.



c	Sample	Identified peptides	Sequence coverage	Phosphorylations
	1	52	83%	55
	2	68	92.2%	39
	3	76	94%	35
	4	60	94%	12
	5	35	78%	4
	6	26	72%	5
	7	23	51.8%	1
	8	38	54.6%	5

Figure 17: Recombinant SBV N nucleoprotein pattern analysis on 12% Acrylamide Tris-Glycine gel and cut strips for subsequent mass spectrometry analysis.

a) On lane 1, SeeBlue® Plus2 Pre-Stained Standard was loaded and on lane 2, 10 µg of recombinant nucleoprotein mixed with loading blue 1x were loaded. After 1 hour of migration under 200 volts, proteins were stained using silver staining.

b) Same picture gel after cutting strips for subsequent mass spectrometry analysis. Arrows indicate the place where strips were taken. Number refers to samples name identified in mass spectrometry analysis.

c) Table shows the sample number that was cut on the gel, the number of mass spectrometry identified peptides in this sample, the percentage of sequence coverage obtained in mass spectrometry and the number of putative phosphorylations.

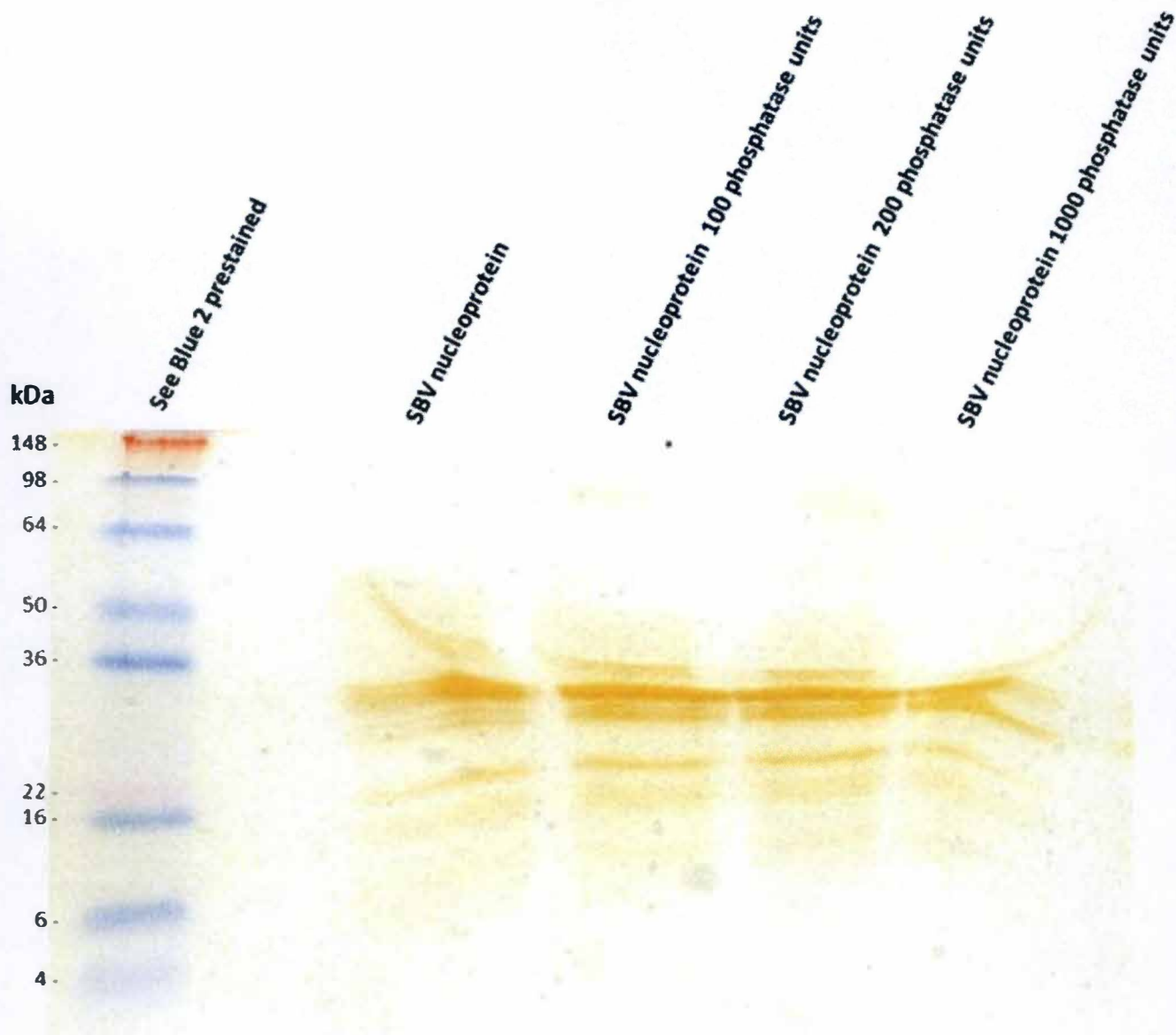


Figure 18: Dephosphorylation assay on recombinant SBV N nucleoprotein. Samples were loaded on 15% Acrylamide Tris-Glycine gel and stained with silver staining.

Lane 1: See Blue 2 prestained molecular standard

Lane 2: 10 μ g of SBV N nucleoprotein mixed with loading blue

Lane 3: 10 μ g of SBV N nucleoprotein treated with 100 units of phosphatase

Lane 4: 10 μ g of SBV N nucleoprotein treated with 200 units of phosphatase

Lane 5: 10 μ g of SBV N nucleoprotein treated with 1000 units of phosphatase

For KC cells, CPE were never observed whatever the MOI, the duration of the infection and SBV viral strain (Figure 16).

Synthesis of SBV N nucleoprotein in baculovirus expression system

To investigate on SBV amplification ratio differences between KC and BHK-21 and on SBV induced CPE in both cell lines, it was necessary to follow the course of SBV inside the cell and to study its interaction with cellular components. We hypothesized that it could be due to differences in viral factories, in viral replication mechanisms or in viral egress in the two types of cell lines. Therefore, it was decided to produce antibodies raised against SBV N nucleoprotein, the most abundant antigen produced during the replication cycle of any bunyavirus. In order to produce antibodies raised against N nucleoprotein, it was necessary to synthesize a recombinant N nucleoprotein. This step was performed in collaboration with BioX Company.

After cloning SBV N nucleoprotein ORF from SBV Na-1 strain (Coupeau *et al.*, 2003), its sequence was modified by adding tags (His-tag and S-tag) useful for further purification steps. The recombinant ORF was cloned downstream the highly active polyhedrin promoter sequence of baculovirus backbone. The recombinant baculovirus recovered after the Sf9 transfer was used to produce high amount of recombinant SBV N nucleoprotein.

Mass spectrometry analysis of recombinant SBV N nucleoprotein

After production and purification of recombinant SBV N nucleoprotein, it was necessary to analyze it before rabbit immunization. The purity of the protein production was verified by gel electrophoresis in one dimension. To determine the molecular weight of recombinant N nucleoprotein and to assess its purity, tandem mass spectrometric (MS) analysis was performed.

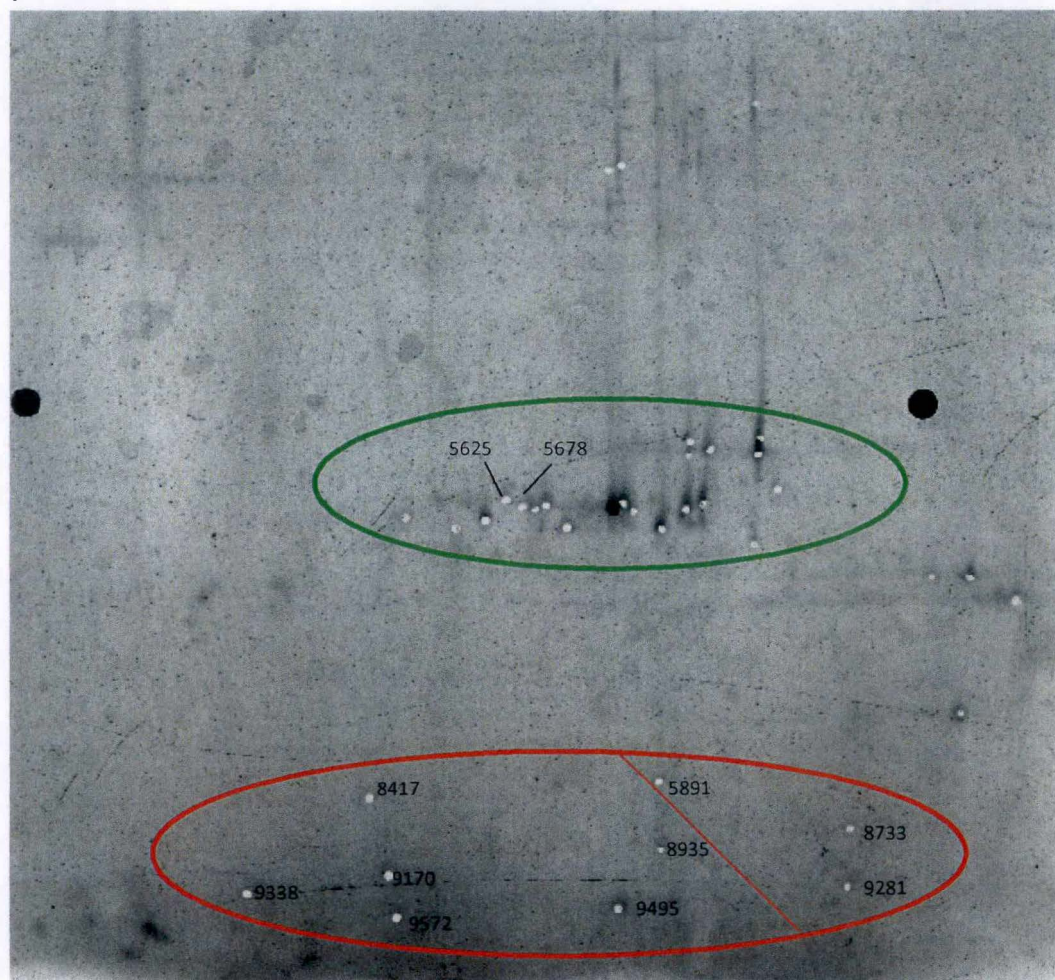
SBV N nucleoprotein was loaded on a 12% acrylamide SDS PAGE gel (Figure 17 a). Results showed 5 differentiated bands between 38 and 4 kDa with 2 large bands presenting a high intensity which could be an overlap of several bands. To identify the proteins in these bands, we used tandem mass spectrometry. 8 bands were cut and send to MS analysis (Figure 17b). Results showed that recombinant SBV N nucleoprotein was pure as no sequenced peptides matched with others proteins than SBV N nucleoprotein in the database.

For high molecular bands, the expected molecular size was 31.5 kDa for the entire tagged sequence of recombinant N nucleoprotein. However, the observed molecular size for the first band on SDS PAGE gel was 37.2 kDa. It was suggested that the molecular shift of 5.7 kDa could be due to post translational phosphorylation modifications of SBV N nucleoprotein. Putative phosphorylations modifications were predicted by MS analysis based on mass differences (as the peptides fragmentation used in MS analysis is not adequate to characterize phosphorylations) (Figure 17c).

For low molecular bands, as sequence coverage was high for all the bands, except for band 8 (Figure 17c), we could not identified a cleavage in N or C-terminal by MS analysis. Therefore, we suggested that these bands could be a mixture of cleaved sequences in different sites or incompletely processed SBV N nucleoprotein forming peptides with very similar masses assembled in the same band on the gel.

pH 3

pH 11



Sequence Coverage	SBV N nucleoprotein (N-term→C-term)	Bio Sample
		9338
		8417
		9170
		9572
		9495
		8935
		5891
		8733
		9281

Figure 19: Two dimension gel analysis of SBV N nucleoprotein before mass spectrometry analysis. 25µg of SBV N nucleoprotein were loaded on a dry strip with a non-linear gradient pH from 3 to 11 before being transferred on the second dimension consisting of a 12.5% acrylamide SDS PAGE gel. Proteins were stained with krypton and 38 spots were taken for mass spectrometry analysis. Table represents analysis of the spots in the red area. Yellow represents part of the sequence which has been covered by sequencing, green represents places of putative modifications as oxidations, carbamidomethyl, phosphorylations and Gln – pyro-Glu. Numbers refers to the spots on the 2D gel that were analyzed by MS. Green area frames peptides distributed among a line and the two spots representing phosphorylated peptides. Red area frames low molecular weight peptides, on the left part N-terminal cleaved peptides and on the right part C-terminal cleaved peptides.

Dephosphorylation assay

As mass spectrometry analysis could only postulate peptides phosphorylation degree based on molecular weight differences, another way of studying phosphorylation was necessary. SBV N nucleoprotein phosphorylation degree was investigated through a dephosphorylation assay.

Three concentration of recombinant phosphorylase were used to remove phosphates of 10 µg of SBV N nucleoprotein. Treated and untreated SBV N nucleoprotein were loaded on a 15% acrylamide SDS Page and stained with silver staining. Results showed that there were no differences observed between treated and untreated conditions whatever the phosphorylase concentration used (Figure 18). However, no positive control was available to assess phosphorylase activity.

2D gel analysis

As the phosphorylation assay on SBV N nucleoprotein was inconsistent, a 2D gel analysis was performed to analyze SBV N nucleoprotein putative phosphorylations and to investigate on the hypothesis of proteins incompletely processed.

Proteins were firstly separated according to their electric charge by isoelectric focusing and secondly according to their molecular weight. This process added a supplementary dimension to sample separation. Thirty eight spots were identified and sent to mass spectrometry analysis. Results showed again that sample was pure, all sequenced peptides matched with SBV N nucleoprotein.

Results showed that there were two putative spots representative of phosphorylated proteins (spot 5978 and 5625, Figure 19). Differences between the other spots were not explained. However, as spots in the middle region of the 2D gel were distributed along a line, it suggested that they were representative of one band on the 1D gel. Differences between these spots should be modifications on these peptides, the most likely still being phosphorylations.

Results showed that small peptides found in the back of 2D gel were proteins with a cleavage in C or N-terminal ends (Figure 19). Those situated in the right area corresponded to peptides with a cleavage in the C-terminal part while peptides from the left back of the gel represented peptides cleaved in N-terminal. Spot 5891 represented the border between N-terminal and C-terminal cleaved peptides on the 2D gel. This suggested that bands of low molecular weight observed on 1D gel analysis were a mix of proteins incompletely processed or cleaved in C-terminal or in N-terminal. In 1D analysis, we were not able to distinguish C-terminal and N-terminal peptides cleavage, because they were not separated according the isoelectric point we obtained high sequence coverage with no gap on the protein extremities.

Production of rabbit polyclonal antibodies raised against SBV N nucleoprotein

Once SBV N nucleoprotein was produced and checked for its purity and its sequence, immunizations were performed to obtain polyclonal antibodies against N nucleoprotein. Two white New Zealand rabbits were immunized with recombinant SBV N nucleoprotein mixed with Stimune adjuvant in three injections sessions separated by intervals of 4 weeks. Before each immunization, blood was sampled by venous puncture from the main ear artery and

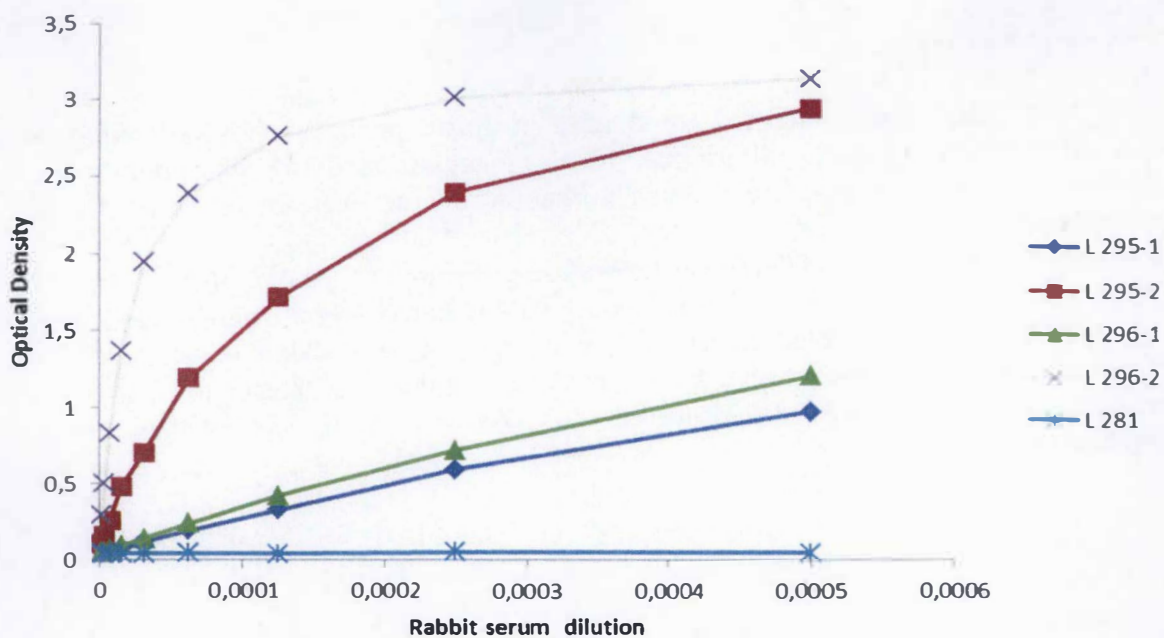
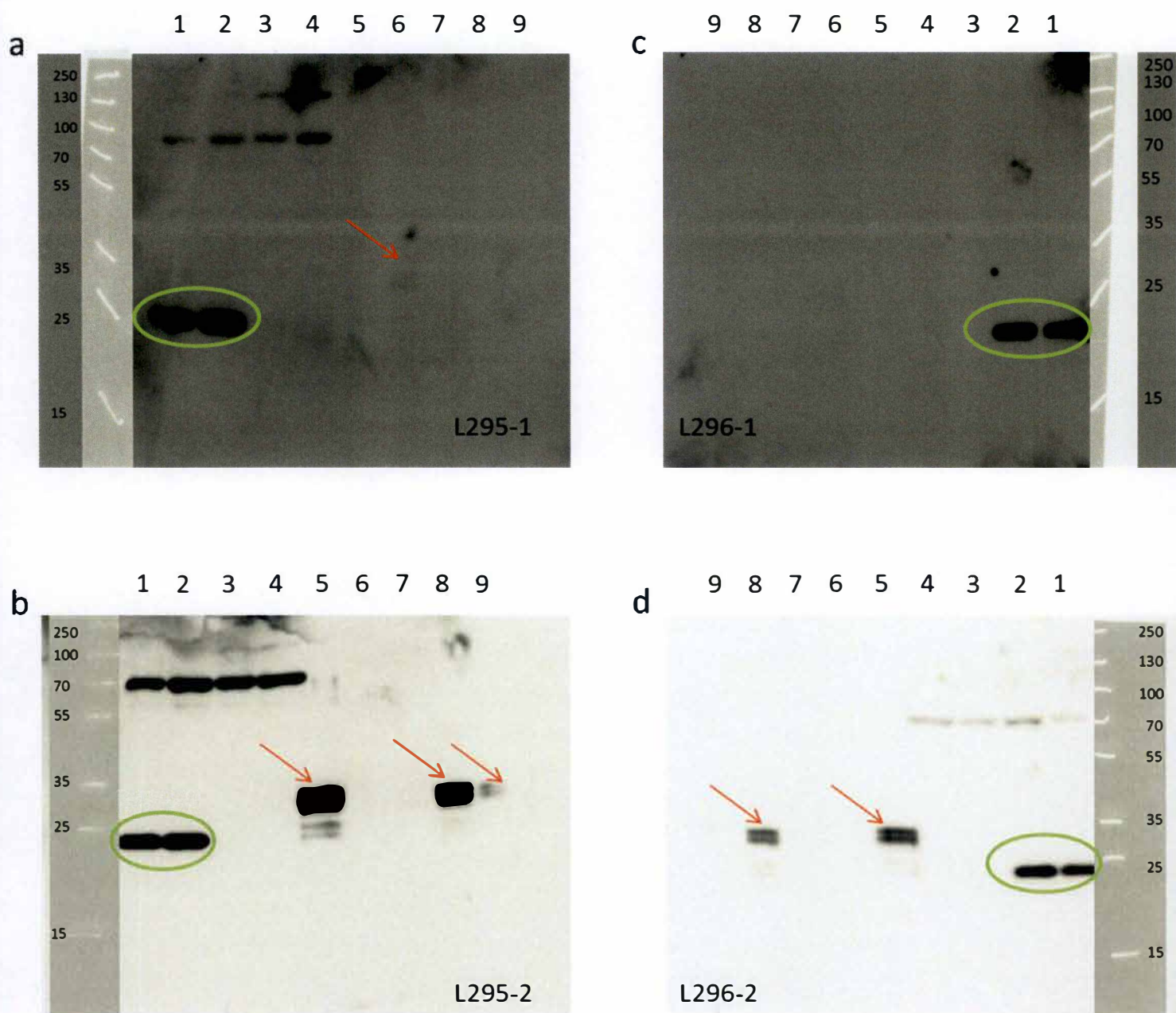


Figure 20: Graphic of indirect Elisa assay on the serum of immunized rabbit with SBV N nucleoprotein, serums are diluted from 1:2000 to 1:512000 following a dilution 2 by 2. In grey, is the optical density in function of serum dilution of the rabbit 295, sampling post first immunization. In red, is the optical density of rabbit 295 serum, sampling post second immunization. In green, is the optical density of rabbit 296 serum, sampling post first immunization. In blue, is the optical density of rabbit 296 serum, sampling post first immunization.



Lanes samples

1. 50,000 infected BHK-21 cells
2. 100,000 infected BHK-21 cells
3. 50,000 uninfected BHK-21 cells
4. 100,000 uninfected BHK-21 cells
5. 4 μ l of SBV nucleoprotein diluted 1:100 = 0.2 μ g
6. 2 μ l of SBV nucleoprotein diluted 1:100 = 0.1 μ g
7. 1 μ l of SBV nucleoprotein diluted 1:100 = 0.05 μ g
8. 2 μ l of SBV nucleoprotein diluted = 0.03 μ g
9. 1 μ l of SBV nucleoprotein diluted = 0.015 μ g

Figure 21: Western blot analysis on serum of rabbit immunized with SBV N nucleoprotein. The same samples were used on each 4 W.B (see box), primary antibody used for revelation is rabbit polyserum immunized with SBV N nucleoprotein a) Rabbit L295 first immunization b) Rabbit L295 second immunization c) Rabbit L296 first immunization d) Rabbit L296 second immunization. Secondary antibody used for revelation is anti-rabbit conjugated with peroxidase. In green is SBV nucleoprotein from natural virus. In red is recombinant SBV N nucleoprotein.

polyserums were isolated. Polyserums reactivity with SBV N nucleoprotein was tested by ELISA, Western Blot and Immunofluorescence assay.

Rabbit serum recognized recombinant SBV N nucleoprotein in ELISA tests.

Enzyme linked immuno sorbent assay (ELISA) is a specific and sensitive method for the detection of specific antibodies. ELISA was used to test the antibodies ability to recognize the immunizing peptide. Microtitre plates were coated with SBV N nucleoprotein. Serums of SBV N nucleoprotein immunized rabbits were added as the tested dilutions (1:2000 to 1:512000). Peroxidase conjugated secondary antibodies were used to reveal the presence of primary antibodies bound to SBV N nucleoprotein. SBV serologically naïve rabbit serum was used as a negative control as well as wells without secondary or primary antibodies. The recognition of the secondary antibodies against SBV N nucleoprotein was tested and was negative (data not showed). There was no OD signal produced by first antibodies utilized without secondary antibodies (data not showed). All the tested conditions were realized in duplicates. Mean optical densities were plotted on a graph according to the tested dilutions (Figure 20).

For the SBV serologically naïve rabbit, OD signal was always situated between 0.042 and 0.054 and was not different from the background OD signal obtained for empty wells. After the first immunization of rabbit 295 and 296, serum recognized SBV N nucleoprotein with a OD signal superior to the OD signal of the negative control until dilution 1:128 000 and 1:512,000 respectively. Maximum OD signals were obtained for smallest dilutions in both rabbits (0.98 for rabbit 295 versus 2.1 for rabbit 296). Rabbit 296 serum recognized SBV N nucleoprotein with a higher OD signal than rabbit 295 for every dilution. After the second immunization of rabbit 295 and rabbit 296, both serums presented an OD signal superior to the OD of the negative control for every dilution (from 1:2000 to 1:512000). Maximum OD signals were obtained for the smallest dilution in both rabbits (2.94 for rabbit 295 versus 3.13 for rabbit 296). Rabbit 296-2 serum reacted with SBV N nucleoprotein with a superior OD signal than rabbit 295-2 serum at every tested dilution. These results showed that the OD was amplified for both rabbits after the second immunization.

Western blot analyses of SBV nucleoprotein in BHK-21 cells

To identify the target proteins of polyclonal antibodies produced in rabbit immunized with the recombinant SBV N nucleoprotein, western blot assay was performed using infected and mock infected BHK-21 cells, and the recombinant SBV N nucleoprotein used for rabbit immunization.

The antibodies of first and second immunization of both rabbits recognized a band of about 23 kDa, representative of N nucleoprotein in SBV infected BHK-21 cells (Figure 21). A band about 70 kDa was recognized in infected and mock infected BHK-21 (lanes 1, 2, 3, 4) cells after the first and second immunization of rabbit 295 and after the second immunization of rabbit 296.

Second immunization serum of both rabbits recognized four bands between 25 and 34 kDa representative of recombinant SBV N nucleoprotein in lane 5 and 8. Serum 295-2 recognized two bands in lane 9 corresponding to recombinant SBV N nucleoprotein.

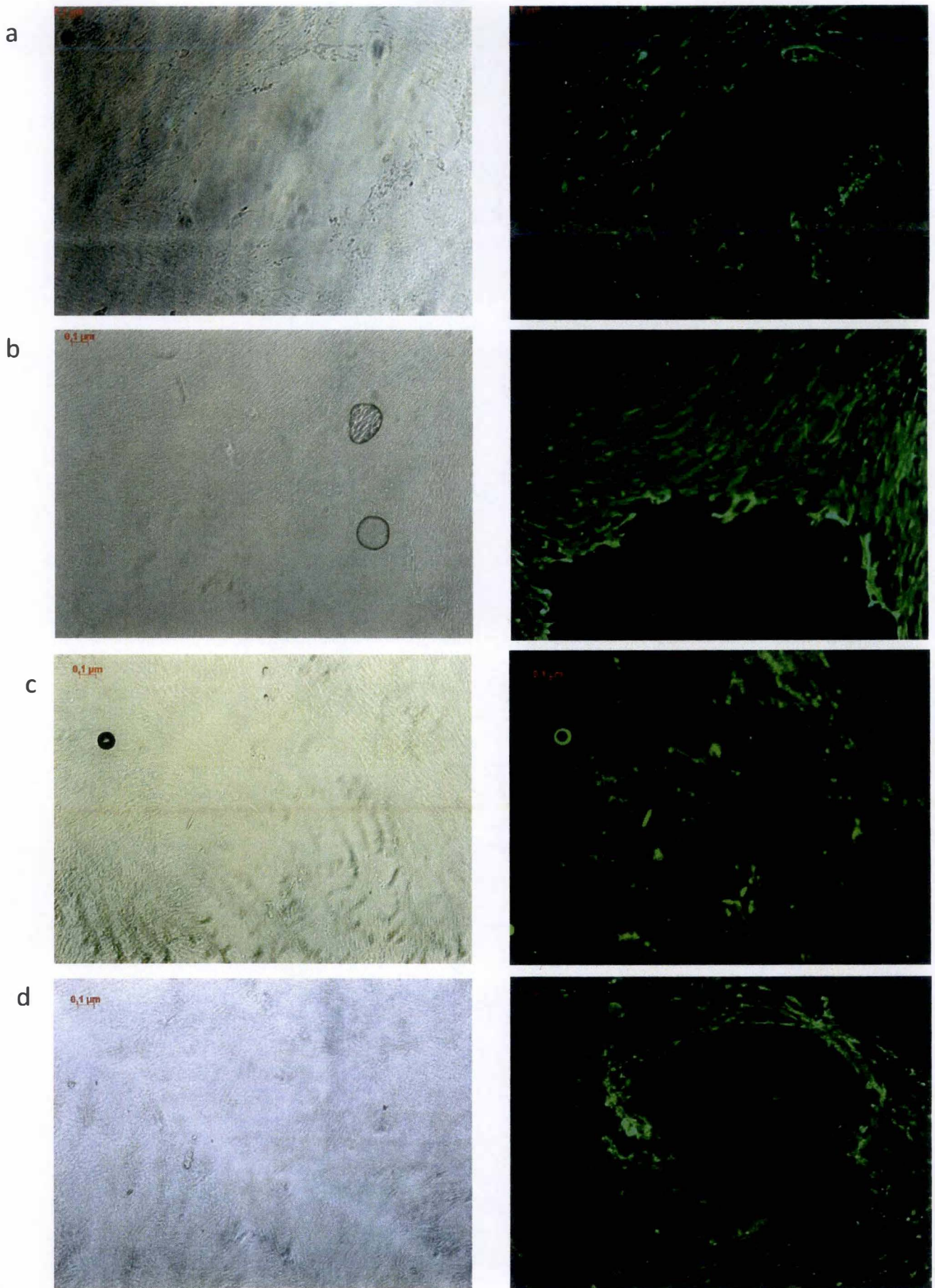
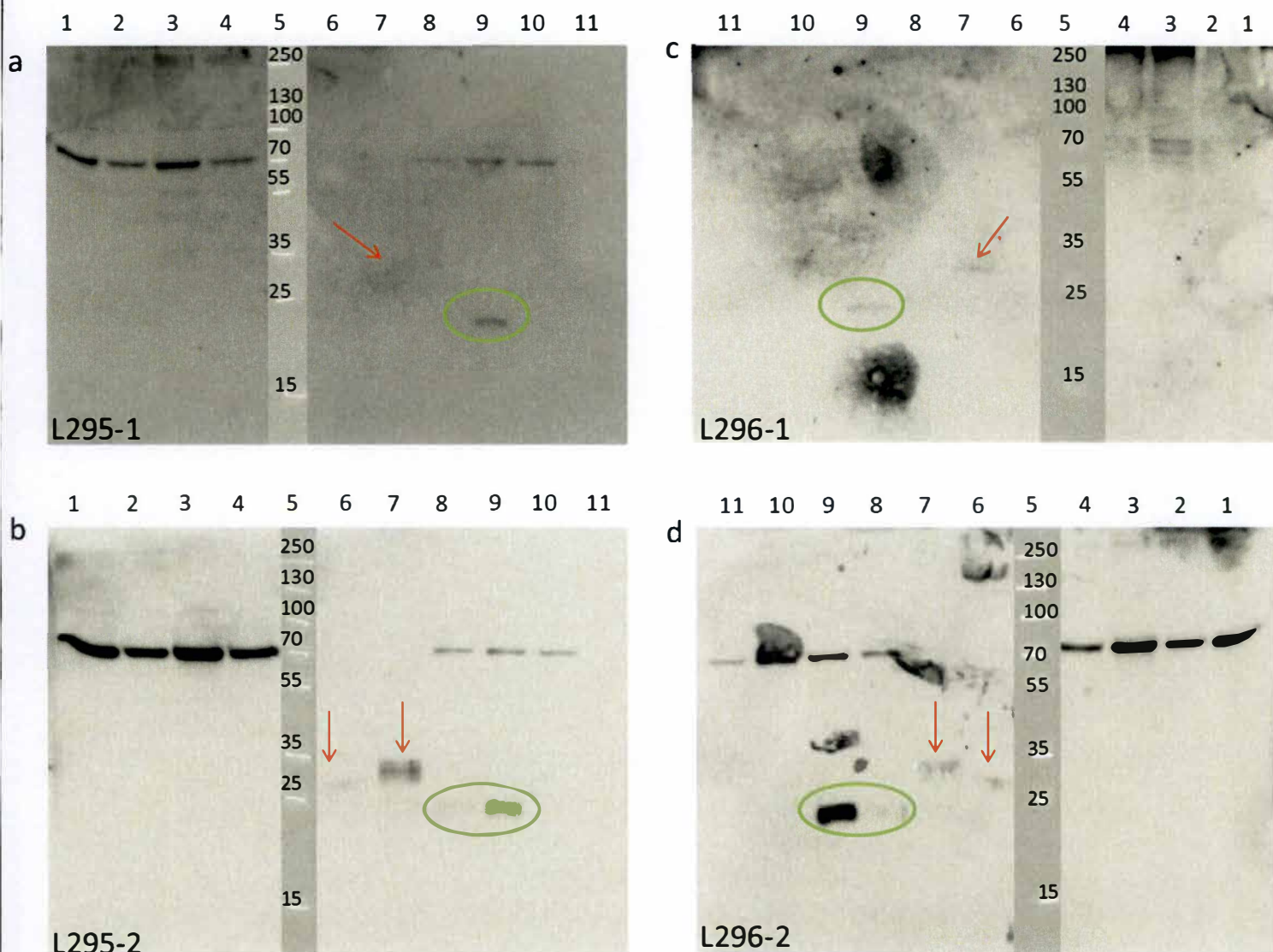


Figure 22: Indirect immunofluorescence assay on BHK-21 cells infected with SBV revealed with rabbit serum (immunized with SBV N nucleoprotein) and anti-rabbit Alexa 488 conjugated secondary antibody. BHK-21 were seeded on cover slips pre-coated with FBS, they were infected with SBV for two hours and then covered with agarose 0.35% for 3 days to isolate viral replication foci. Infected cells were stained with rabbit serum. a) first immunization rabbit 295 b) second immunization rabbit 295 c) first immunization rabbit 296 d) second immunization rabbit 296 and revealed with anti-rabbit secondary antibody conjugated with Alexa 488. Pictures were taken at zoom 40x once in phase contrast (left column) and once with epifluorescence (right



Lanes samples

1. 400,000 un infected KC cells
2. 200,000 uninfected KC cells
3. 400,000 infected KC cells
4. 200,000 infected KC cells
5. Pageruler
6. 2 μ l of SBV nucleoprotein diluted = 0.03 μ g
7. 2 μ l of SBV nucleoprotein diluted 1:100 = 0.1 μ g
8. 10,000 infected BHK-21 cells
9. 20,000 infected BHK-21 cells
10. 10,000 uninfected BHK-21 cells
11. 20,000 uninfected BHK-21 cells

Figure 23: Western blot analysis on serum of rabbit immunized with SBV N nucleoprotein. The same samples were used on each 4 W.B (see box), primary antibody used for revelation is rabbit polyserum immunized with SBV N nucleoprotein a) Rabbit L295 first immunization b) Rabbit L295 second immunization c) Rabbit L296 first immunization d) Rabbit L296 second immunization. Secondary antibody used for revelation is anti-rabbit conjugated with peroxidase. In green is SBV nucleoprotein from natural virus. In red is recombinant SBV N nucleoprotein

No other bands were highly recognized by rabbit serums.

Antibodies interaction with SBV infected BHK-21 cells through indirect immunofluorescence

To assess if antibodies raised against recombinant SBV N protein recognized viral native N protein, the reactivity of the rabbit antibodies was tested by an indirect immunofluorescence assay on SBV infected BHK-21 cells.

BHK-21 cells were infected with SBV. Infected monolayers were incubated in the presence of semi-solid agar medium to induce the formation of viral foci. When isolated infection foci were observed, cells were fixed and then incubated with rabbit serum. Secondary anti-rabbit antibodies conjugated with Alexa 488 were used to reveal the presence of rabbit antibodies bound to N nucleoprotein. Therefore, green fluorescence was indicative of the interaction of serum antibodies with SBV N nucleoprotein.

Pictures of isolated replication foci were taken once in epi-fluorescence and once in phase contrast (Figure 22). Results showed that the four rabbit serum samples were reactive with infected BHK-21 cells although immuno-staining intensity varied between serums. On the four epi-fluorescence pictures, infected cells (in green) surrounded a black hole representative of cells lysed by viral replication. The cells around the black hole are flattened and began to detach. Away from the black hole, there are less green cells and on epi-fluorescence picture, a black area is observed which is representative of uninfected cells that we can see on phase contrast picture.

Rabbit 295 serum showed a better signal after the second immunization than after the first immunization, similar results were observed for rabbit 296 serums.

There was no immuno-reactivity of the rabbit antibodies with uninfected cells as indicated by cells around SBV replication foci which were not immuno-stained. In the same manner, uninfected cells were used as negative control and were not stained by the four rabbit serum (data not showed). Limited background signal was observed for primary antibodies used alone (data not showed). Secondary antibodies did not recognize SBV or cellular components (data not showed).

Western blot analyses of SBV nucleoprotein in KC cells

To determine the reactivity of polyclonal antibodies with its corresponding protein, western blot assay was performed using SBV infected KC cells and recombinant SBV N nucleoprotein. Anti-rabbit peroxidase conjugated secondary antibodies were used to show immuno-reactivity of rabbit antibodies. It is important to note that results from rabbit 296 first immunization were hazardous to interpret due to a problem during membrane transfer.

Results (Figure 23) showed that antibodies obtained after the first and second immunizations of rabbit 295 and the second immunization of rabbit 296 recognized a band around 70 kDa (lanes 1 to 4), in SBV infected and uninfected KC cells (similarly to what was observed in BHK-21 cells). The band corresponding to native SBV N nucleoprotein (23kDa) was not observed in infected KC cells (lanes 3, 4) by any of the four rabbit serum.

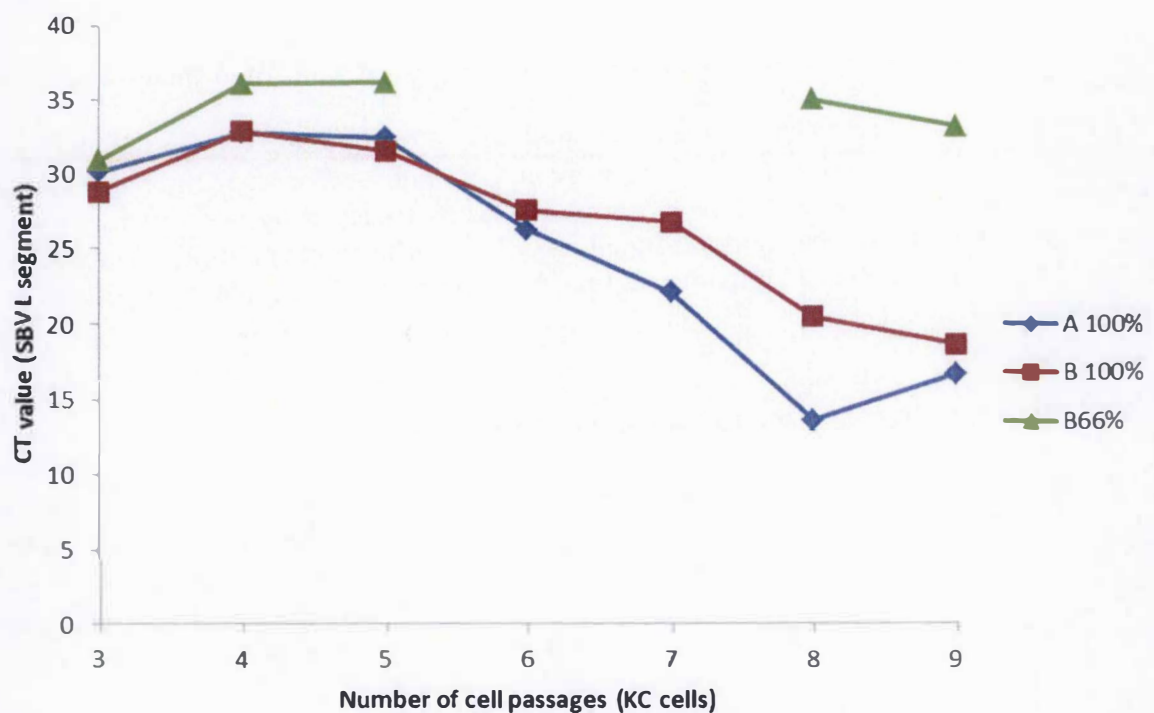
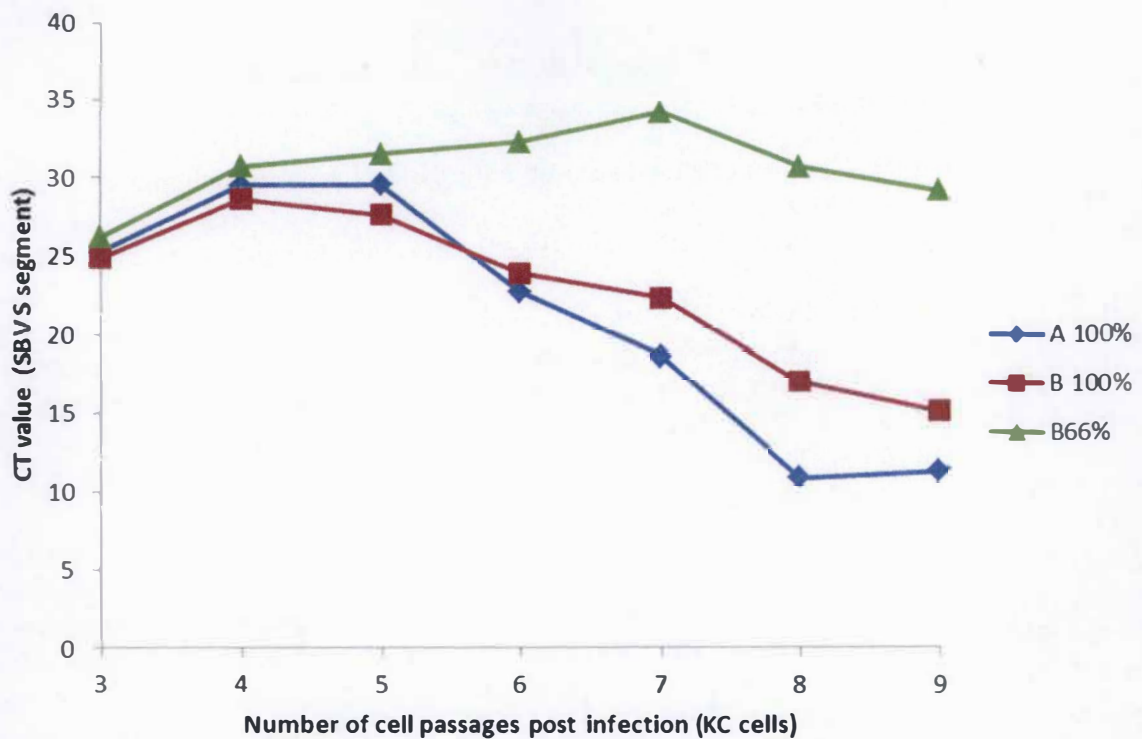


Figure 24 Graphs showing the evolution of CT values obtained in RT-RT qPCR one step analysis of SBV L (a) and S segment (b) through successive cellular passages of initially infected KC cells. The condition A represents a pool of KC cells that were infected at a low cellular density while condition B represents KC cells infected at high cellular density. Green curve represent B condition cells that were re-seeded (when they reached cellular confluence) following 2/3 infected cells, 1/3 uninfected cells ratio. Red curve represents B condition cells that were re-seeded at cellular confluence following a ratio of 100% infected cells. Blue curve represents A condition cells that were re-seeded at cellular confluence following a ratio of 100% infected cells. Total RNA was extracted from cellular suspension between cellular passage 3 and 9. RNA was analyzed in qPCR one step SBV for L and S segment.

Bands between 25 and 34 kDa (lanes 6, 7) representative of recombinant SBV N nucleoprotein were recognized by second immunization serum of both rabbits and slightly recognized by first immunization serum of both rabbits.

No other bands were highly recognized by SBV N nucleoproteins serum rabbits issued from the second immunization while other bands were slightly recognized by serums issued from the first immunization.

BHK-21 infected and uninfected cells were used as positive control in lanes 8 to 11 and were recognized by four serums.

Antibodies interaction with SBV infected KC cells through indirect immunofluorescence

Immunofluorescence was performed on SBV infected KC cells with four rabbit serum. KC cells were seeded on cover slips and infected with SBV for three days. After what, cells were fixed, permeabilized, incubated with rabbit serum immunized with SBV N nucleoprotein and then with anti- rabbit antibodies Alexa 488 conjugated.

KC cells cover slips were observed in fluorescence microscopy. As KC cells were hard to growth and were loosely attached on glass cover slips, complete cellular monolayer was hard to obtain. Moreover, isolated SBV replication foci were not observed because KC cells did not present CPE. Therefore results were hard to interpret.

SBV adaptation after successive passages in KC cell cultures

As amplifications ratios were poor in KC cells compared with BHK-21 cells, we postulated that it could be due to RNAi response in KC cells. We considered that if this process limited SBV replication rates in KC cells, it should exert a selective pressure on the virus and maybe lead to the selection of mutant viruses that can escape RNAi. To test this hypothesis, we performed long term infection in KC cells over 9 cellular passages during 5 weeks. KC cells were infected with SBV BH80/11-4 and cellular suspensions were collected after every cellular passage. Total RNA was extracted and tested in RT-RT one step qPCR for detecting and quantifying S and L SBV segments.

Three infected conditions were performed; A 100% represented a condition of low cell density infected with SBV that were passed at 1:6 dilution when they reached cellular confluence, B 100% represented a condition of high density cells initially infected with SBV passed at 1:6 dilutions when they reached cellular confluence, B66% comes from the same infected cells batch (as B100%) but in B66% conditions these cells were passed following a ratio of 0.66/0.33 respectively for infected/non infected cells.

Viral adaptation did not occur in the condition B66%. Initial CT values observed at passage 3 increased slightly and stabilized at high level (33 for L; 20 for S) corresponding with low replication level of SBV. Initial CT value was obtained for passage 3 (30.92), after what CT value raised until passage 5 (36.16), before a decrease between passage 8 (35) and 9 (33.16).

For A100% and B100% condition, both segment analyses presented similar evolution through successive cellular passages with an increase between passages 3 to 5 before a high decrease of the CT values until passage 9. This suggested that a mutation change occurred leading to the selection of an adapted virus which presented a better fitness. A100% presented lowest CT values after passage 6 for both segment analyses.

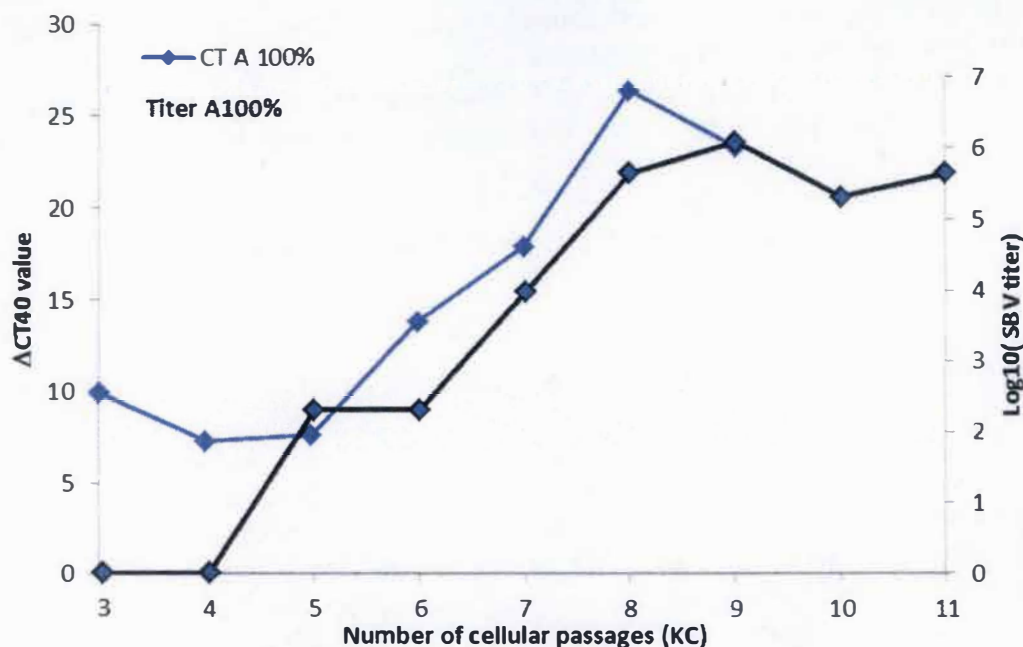


Figure 25: Graph showing SBV viral titer evolution in parallel with $\Delta 40$ CT (L segment) value for conditions A100% through successive cellular passage of KC cells.

Black curve represent viral titer evolution and refers to the right vertical axis. Cellular suspension was sampled after cellular passage (3 to 11) of infected KC cells (condition A100%). Cellular suspension was incubated on BHK-21 cells for two hours, BHK-21 were incubated for three days in their growth culture condition. Cytopathic effects were observed and TCID₅₀ (tissues culture infecting dose) was calculated according to reed and Muench method.

Blue curve represents $\Delta 40$ CT value of A100% conditions evolution and refers to the left vertical axis. CT value for A100% conditions were subtracted from 40 to obtain the $\Delta 40$.

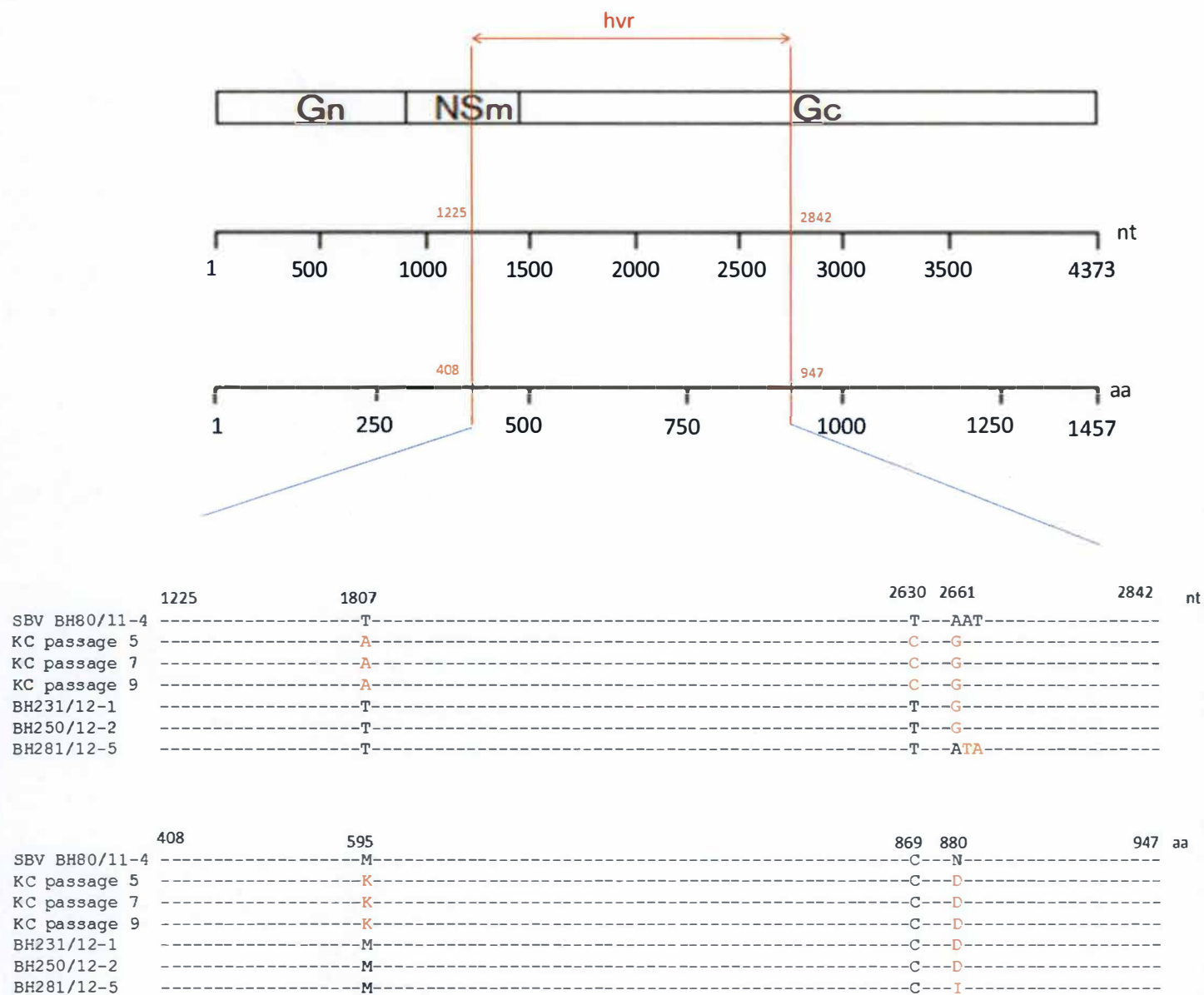


Figure 26: Alignment of SBV hyper-variable region (M segment) nucleotide and amino acid sequences. Hyper variable region (nt 1225-2842) is situated on M segment between NSM and GC ORF. SBV BH80/11-4 is the viral strain we used to perform long term infections in KC cells. KC passage 5, 7, 9 represent sequences of viruses samples after cellular passage (5, 7, and 9) in KC cells long-term infection. BH231/12-1, BH250/12-2, BH281/12-5 sequences comes from Fischer *et al.*, 2013 paper and represent, viruses sampled in brain from lamb fetus, cox and lamb respectively. Nucleotidic (1225-2842) and amino acid sequences (408-947) we aligned. Mutations compare with SBV BH80/11-4 are in red while nucleotides/ amino acids unchanged are in black.

Globally, results showed that S qPCR was more sensitive than L qPCR with a diminution of 3 to 5 CT value for every sample. This could be explained by the higher level of expression for the S segment compared to the L segment. Indeed, S segment codes N nucleoprotein that is needed in a higher rate than L polymerase which is coded by L segment.

Titration of successive cellular passage in KC cells long term infection

To assess if viruses tested in RT-RT one step qPCR were still alive and infectious, titration on BHK-21 cells were performed. Condition A100% was chosen to be tested because it presented the best viral amplification in qPCR results. Serial dilutions of each supernatant were tested in BHK-21 cell culture prepared for determining the TCID₅₀ values. Cytopathic effects were observed and TCID₅₀ was calculated according to Reed and Muench method. Data was plotted using Log10 (TCID₅₀) in terms of successive cellular passages (Figure 25).

For passage 3 and 4, no CPE were observed in BHK-21, indicating that these initial steps of KC infection did not produce any infectious particles. The first viral titer was obtained for passage 5 ($10^{2.03}$ TCID₅₀/ml), after what viral titers rose sharply from passages 6 to 9, to reach an infectious supernatant at $10^{6.07}$ TCID₅₀/ml. The viral titer slightly decreased at passage 10 and 11.

In parallel of these data, Δ CT40 of A100% condition (L segment analysis) was plotted to compare viral amplification ratio assessed by qPCR and titration. Infectious virus appeared (after passage 4, titration) one cellular passage before viruses started to amplify (after passage 5, qPCR). Then, titer curve and Δ CT40 curve rose sharply and while Δ CT40 decreased after cellular passage 8, titer only decreased after cellular passage 9.

Evolution of SBV S and L segment sequences in long term infection of KC cells

During long term infection of KC cells, a change in the amplification rate was observed after passage 5 for A100% and B100% conditions until passage 9. As CT values were better for A100% than B100%, we decided to study A100% conditions. Small segment and hyper variable region (M segment) of passage 5, 7 and 9 were sequenced to assess sequence evolution before, during and after this abrupt change in amplification rate.

Consensus sequences of the five samples of cellular passages 5, 7, 9 were aligned with the sequence of the viral strain initially used for infections (Figure 26). Results showed that for S segments, no mutations were observed between consensus sequences and reference sequence. For hvr1 (first part of hvr on M segment) one mutation in position 1807 (nt) was found, A instead T. This mutation was found in the three cellular passages tested. For hvr2 (second part of hvr on M segment), two mutations were found, a C instead T (nt 2630) and a G instead A (nt 2661). These mutations were maintained for every cellular passage studied.

Impact of these three mutations on amino acid sequence was investigated. Mutation at position 1807 led to a change from a methionine (M) to a lysine (K) for three cellular passages. Mutation at position 2631 did not lead to amino acid change. Mutation at position 2660 led to a change from asparagine (N) to an aspartic acid (D).

Discussion, conclusion,
perspectives

Discussion

SBV is a new arbovirus that emerged in 2011. Since then, its relatedness with 2 ruminant Simbuviruses, Sathuperi virus and Shamonda virus has been established. SBV origin is still unknown although different studies postulated that SBV is the result of reassortment process. It was also demonstrated that SBV was transmitted by *Culicoides*. However, no studies have investigated on the relation between these three Simbuviruses and their vectors. For that purpose, growth kinetics of SBV in BHK-21 cells and KC cells derived from *Culicoides sonorensis* were performed. Amplification ratio was higher in mammalian cells than in *Culicoides* cells. Moreover, these last did not show cytopathic damages after SBV infection unlike BHK-21 cells. It was also noticed that infection with SBV showed poor repeatability in KC cells and that infection with a stronger MOI was associated with a better amplification.

To investigate on the origin of these differences observed in BHK-21 and KC cells, antibodies against SBV were produced in order to follow the course of SBV inside the cell and to study its interaction with cellular components. SBV N nucleoprotein was chosen as a target to develop polyclonal antibodies due to its high expression level in infected cells. Synthesis of recombinant N nucleoprotein was conducted by BioX Company. Before immunization, recombinant SBV N nucleoprotein was checked. Results showed a potential high degree of phosphorylation. Therefore, a de-phosphorylation assay and a 2D gel analysis were performed to investigate on these putative phosphorylations. Results also showed that SBV N nucleoprotein was pure. Therefore, rabbit immunization was performed.

Polyserum obtained after first and second immunization of two rabbits were tested by ELISA, immunofluorescence and Western blot assays. Their reactivity with native and recombinant SBV nucleoprotein was demonstrated. Their specificity and their titer were assessed in ELISA. In BHK-21 western-blot assay, antibodies were specific to both forms of SBV N nucleoprotein. Similar assays were developed in KC cells, but native SBV N nucleoprotein was not recognized by antibodies maybe due to the poor amplification ratio in this cell line. In immunofluorescence assay, antibodies specificity against BHK-21 infected cells was demonstrated.

To solve the problem of poor amplification ratio in KC cells which could be responsible of the poor results obtained in western blot assay and in immunofluorescence assay, it was decided to perform a long term infection in KC cells. We postulated that through successive cellular passages in KC cells, SBV would adapt to this cell line and present a higher rate of replication. Viral amplification rate and titer was monitored through successive cellular passage by performing qPCR targeting on the L and S segment of SBV and by titration on BHK-21 cells. Results showed that viruses were still alive and infectious as viral titers could be obtained after passage 3. Results also showed that after cellular passage 5, CT value decreased until rates that had never been observed previously, suggesting a viral adaptation. Therefore, S segment and the hyper-variable region on M segment were sequenced to assess which genetic changes were associated with this viral adaptation.

Interaction between simbuviruses and *culicoides* cells

As a first approach, SBV interaction with mammalian (BHK-21) and insect cells (KC) was studied. Comparison of SBV amplification ratio in KC and BHK-21 cells showed a sharp difference in the rates of amplifications. For BHK-21, SBV amplification ratio was higher than in KC cells. This difference could be explained by the species from which cell lines have been developed. KC cells come from *culicoides* that have been identified as SBV vectors

while BHK-21 cells comes from mammals. Although it has not been described specifically for SBV, the life cycle of a virus transmitted by arthropods vectors is the following: first, the vector (female) bites a susceptible host, the virus infects the host and replicates in it. Then, an arthropod vector takes a blood meal on the infected host and ingests viraemic blood. As the ingested food is blood, a sphincter muscle of the diverticulum contract and the blood is directed to the hind part of the midgut (Megahed, 1956 cited in Mellor *et al.*, 2000). Once there, viruses attach to gut cells, enters in the cell and are released at the basolateral pole, enter haemocoel, disseminate through it and infect secondary organs as salivary glands or ovaries/testes (Mellor *et al.*, 2000). The virus replicates in the vector and the transmission cycle starts again when the vector infects a susceptible host. Yet, if the virus amplifies with a high rate that is harmful for the vector, it will diminish the number of vectors and so decreases the number of chances of the virus to infect a serologically naïve host and *in fine* leads to the clearance of the virus. That could explain why SBV showed a limited replication rate in insect cells compared to mammalian cells.

The difference observed in the rate of growth between the kinetics of two different MOI (0.01 and 0.5) could be explained by the nature of the cell response engaged to counter the virus replication. Nothing is known about the interactions of SBV with vectors immune and/or cell response. However it has been showed by Schnettler *et al.*, 2013 that RNAi response can be induced in KC cells by viral infection with SBV. After infections of KC cells, Schnettler *et al* isolated small RNAs and sequenced them. They found that there was genomic and antigenomic small RNAs and a majority of them were 21 nucleotide lengths and mapped to each SBV segments. Schnettler *et al* suggested that there should be a dsRNA intermediate during replication that could induce the RNAi response in KC cells infected with SBV. However, as the entire genome of *Culicoides* is unknown, we can only suggest that there should be orthologs genes of Dicer and Agonaute proteins that are involved in the RNAi pathway presents in *Culicoides*. We can hypothesize that under a number of viruses per cells, the RNAi pathway is efficient and counters viral replication rate. Beyond this threshold, the RNAi pathway is overcome and viruses replicate with a higher rate.

Paradoxally, arthropods do not have interferon response of vertebrates but control efficiently arbovirus infection. This efficient control is due to peculiar innate immune response (Fragkoudis *et al.*, 2009 cited in Lopez-Montero *et al.*, 2011) and antimicrobial immune pathway. We could hypothesize that this mechanisms in *Culicoides* maintains a low rate of growth for SBV. The poor amplification rate of SBV in KC cells may be also due to temperature effect. Indeed, temperature could affect the ability of the vectors to modulate viral replication within the cells (Hardy *et al.*, 1983 cited in Mellor 2000) and so has an impact on virogenesis and is specific to each vector-virus couple (Mellor *et al.*, 2000). At elevated temperature, virogenesis is faster than at low temperature. It is likely that 25°C is the optimal temperature for KC cells growth but not for their permissiveness to SBV replication.

The poor amplifications ratios we obtained in KC cells have been previously observed in other studies (Schnettler *et al.*, 2013; Beer M, FLI, personal communication). It could be due to RNAi response but it could also be due to persistent infection of KC cell line with BTV (Nayduch *et al.*, 2013). Indeed, the cell line we used has been showed to be persistently infected by a latent BTV. This persistent infection can limit viral amplification of SBV in KC cells due to inhibition caused by the process of superinfection. Therefore, we could use other cell lines derived from *Culicoides* (unfortunately hard to obtain from labs using them) or develop our own *Culicoides* cell lines which will take around one year. We could also use other cells lines of other arthropods such as mosquito C6/36 cell lines that have been showed

to support a good amplification rate of SBV (Martin Beer, FLI personal communication). Moreover, this cell line has been shown to be defective in Dicer 2-based RNAi response (Morazzani *et al.*, 2012 cited in Szemiel *et al.*, 2012) supporting the hypothesis of limited amplification ratio in KC cells due to RNAi response.

Cytopathic effects

All infection experiments performed in KC and BHK-21 were monitored for CPE. Results showed that none of the infection experiments performed in KC cells presented cytopathic effects unlike BHK-21 cells. Absence of CPE in KC cells is consistent with what has been previously observed for KC cells infected with BTV (Mecham 2006).

The explanation could reside in the way of releasing virion from infected cells. It was observed for mammalian cells that virions are released from the infected cells by budding and by rupture of cell membrane while in insect cells virions are released by budding without cellular damage (Fu *et al.*, 1995 cited in Mellor *et al.*, 2000). Extrusion of the viruses is accompanied with cells surface debris indicating cell damages that could play a major role in lysis and death of infected BHK-21 cells.

In addition, it has been observed during BUNV infection in mosquito cells, that nucleocapsid and RNA polymerase viral proteins were sequestered into membrane vesicles. It has been suggested that this trapping of essential proteins for viral replication and assembly could protect cells from deleterious effects that lead to death in mammalian cells (Lopez-Montero *et al.*, 2011). Moreover, BUNV NSs protein causes the shut off of host cell protein synthesis and the inhibition of IFN-1 production in mammalian cells exclusively (Bridgen *et al.*, 2001 cited in Lopez-Montero *et al.*, 2011). As SBV possesses a NSs protein, we could hypothesize that in BHK-21, SBV shuts off protein synthesis and lead to cell death. However, in KC cells NSs did not shut off protein synthesis and therefore did not lead to cytopathic effects in KC cells.

Moreover, it was observed in mammalian cells, that BUNVs accumulate in the Golgi causing a swelling and a fragmentation of the Golgi and disrupting the secretory pathway (Salanueva *et al.*, 2003 cited in Lopez-Montero *et al.*, 2011). Yet, in mosquito cells infected with BUNV, there was no perturbation in the Golgi stacks. BUNV assembly was restricted to peripheral Golgi stacks and did not accumulate in it. We could hypothesize that the same phenomenon exists for KC cells and could explain the absence of cytopathic effect after SBV infection compared with BHK-21. To investigate on viral egress and on accumulation in Golgi stacks, confocal analysis should be performed after simultaneous immuno-staining of N nucleoprotein and cellular components in the both cell lines.

Recombinant SBV N nucleoprotein analysis

We isolated, sequenced and cloned the N gene from an SBV isolate and expressed it in *baculoviruses expression system*. Before rabbit immunization protein was checked.

Results showed that recombinant protein was pure, however many peptides of different molecular weight were observed suggesting that there was uncompletely processed recombinant nucleoprotein. Moreover peptides with a molecular weight superior to what was expected suggested that there should be post translationnal modifications on the recombinant proteins. Mass spectrometry analyses suggested that phosphorylations modifications were plausible. However, as mass spectrometry could only postulate phosphorylation modification based on molecular weight difference, we needed to confirm it. Therefore, it was decided to perform a dephosphorylation assay.

Dephosphorylation assay

Recombinant nucleoprotein was treated with dephosphorylase following three concentrations, samples were loaded on a SDS PAGE and stained with Silver stain to analyze putative phosphorylation of nucleoprotein.

No differences between phosphorylase treated and untreated SBV N nucleoprotein was observed and this whatever the phosphorylase concentration used. As we have no positive control to assess of the dephosphorylase activity, we cannot assess if there were no phosphorylation on SBV N nucleoprotein or if the phosphatase did not work. Therefore we need a phosphorylated protein (ex: phosphorylated myelin basic protein used in the quality control of dephosphorylase) which have been previously tested with this dephosphorylase and which presented positive results to this enzyme. We could also study phosphorylation degree using another method, for example ProQ Diamond which is a specific staining of protein phosphorylations.

2D gel analysis

As dephosphorylation assay results were inconsistent, a 2D gel electrophoresis was performed in order to analyze peptides separated according to their isoelectric point and their molecular weight. The discrimination is better than the 1D gel electrophoresis. Results showed again that sample was pure, all sequenced peptides only matched with SBV N nucleoprotein. Results did not allow us to know more about phosphorylation degree. Indeed, only two spots were suggested to represent phosphorylated proteins. However, during fragmentations of peptides by collision-induced dissociation (CID), peptidic bonds are cut and phosphates are removed from peptides. Therefore, CID is not appropriated to study proteins phosphorylation degree and the hypothesis of phosphorylations modifications is still plausible. We could use ETD (Electron-transfer dissociation) to fragment peptides in collision cell which is a softer fragmentation technique that conserves protein phosphates. We could also use phosphopeptides purification step before mass spectrometry analysis which is specifically directed against phosphates.

Otherwise, results showed that small peptides found in the back of 2D gel were proteins with a cleavage in C or N-terminal extremities, supporting the hypothesis of cleaved proteins. In addition, if we consider the pI (isoelectric point) of the complete protein (7.19), a cleavage in N-terminal (until aa 120) would lead to a pI of 6.7 while a cleavage in C-terminal (from aa 120) would lead to a pI of 7.95. These results are consistent with the observations made on 2D gel analysis where N-terminal cleaved peptides are situated on the left of the gel (decrease of I.P) while C-terminal cleaved peptides are situated on the right of the gel (increase of pI).

As results did not suggest that nucleoprotein modifications could interfere with the development of antibodies directed against nucleoprotein, investigations about phosphorylation degree of the protein were stopped and production of antibodies was started.

SBV N nucleoprotein antibodies analyses

The purified recombinant N protein was used to immunize rabbits in order to produce polyclonal antibodies directed against SBV N nucleoprotein. The characteristics of these antibodies were studied in ELISA, western blot and immunofluorescence assay.

ELISA assay

Second immunization of rabbits led to an amplification of antibodies titer in comparison with first immunization. All the negative controls were good, secondary antibodies were specific to primary antibodies and did not bind to SBV N nucleoprotein. Primary antibodies did not interact with spectrophotometer reading at 450 nm. Serum of the serologically naïve rabbit presented a very low OD signal suggesting that the antibodies directed against SBV N nucleoprotein were induced by immunization process.

Therefore, the developed polyclonal antibodies showed good sensitivity and specificity and could be used in serological diagnosis (ex: sandwich ELISA).

Western blot assay with mammalian and insects cells

In a first time, western blot assay were performed using infected BHK-21 cells which supported a high replication rate of SBV. As results were conclusive for assay performed in BHK-21 cells, it was decided to realize similar assay in KC cells. Western blot analyses were only qualitative and assessed recognition of the SBV N nucleoprotein / contaminants by rabbit antibodies.

Results showed that all rabbit serum recognized a band around 23 kDa in BHK-21 cells but not in KC cells. As this band was not recognized in uninfected BHK-21/ KC cells, it supported the fact that this 23 kDa band was representative of SBV N nucleoprotein. The fact that any of the four rabbit serum recognized native SBV N nucleoprotein in infected KC cells could be due to the poor amplification ratio of SBV in this cell line.

Rabbit serum also recognized bands around 28-36 kDa, representative of recombinant SBV N nucleoprotein. These results suggested that the four rabbit serum contained antibodies directed against recombinant and native SBV N nucleoprotein. The molecular weight switch between the bands corresponding to native and recombinant SBV N nucleoprotein could be explained by the presence of His-tag and S-tag and putative phosphorylation modifications in the recombinant form. The four bands observed on western blot assay corresponding to recombinant nucleoprotein are consistent with observations made on recombinant nucleoprotein SDS PAGE analyses. Indeed, on SDS PAGE, several bands corresponding to nucleoprotein were observed. After mass spectrometry analysis, it was confirmed that all these bands were representative of SBV N nucleoprotein.

Apart a band around 70 kDa in infected and uninfected KC and BHK-21 cells lanes, no others bands were highly recognized by the antibodies. This 70 kDa bands have not been identified yet. As this band was recognized in infected and uninfected cells it was not induced by SBV infection. Therefore, it could be a cellular component of BHK-21/KC cells or a component of the cell media in which cells were grown. It might correspond to the Bovin serum Albumin (BSA) that has a predicted molecular mass of 66.5 kDa.

Globally, it appeared that serum of the second immunization presented a better signal suggesting a higher titer of antibodies directed against SBV N nucleoprotein. That was consistent with the results obtained in ELISA. Moreover, second immunization antibodies appeared to be more specific than first immunization as few bands (instead bands around 70kDa, and SBV N nucleoprotein bands) were slightly recognized by first immunizations serums while it was not the case for second immunization serums bands. This suggested that serums issued from second immunization were more specific and/or more concentrated in SBV N nucleoprotein antibodies.

These results confirmed that developed antibodies directed against SBV N nucleoprotein present the necessary qualities to perform SBV amplification kinetics through time and to study interaction with proteins by co-immunoprecipitation assay.

Immunofluorescence assay in mammalian and insect cells

Immunofluorescence assay were performed on KC and BHK-21 cells to assess if antibodies recognized specifically infected cells and did not reacted with uninfected cells. Antibodies from the four tested rabbit serum reacted with infected BHK-21 cells. Indeed, infected BHK-21 cells were immuno-stained while uninfected BHK-21 cells were not suggesting that antibodies reacted specifically with native SBV nucleoprotein. Immuno-staining intensity varied between serums. Indeed, signal intensity was better for the second immunization serum of both rabbits suggesting that antibodies titer was higher after second immunization than after first immunization similarly to previous observations made in western blot and ELISA assay.

As results were conclusive for BHK-21 assay, it was decided to perform immunofluorescence on KC cells. However, as this cell line did not present CPE, it was not possible to obtain isolated replication foci of SBV. Moreover, as this cell line is slightly adhesive, cells did not supported well immuno-staining protocol and many of them detached of the glass cover slip. Therefore, results were hard to be interpreted. Further experiments are needed with a viral strain presenting better amplification ratio or with *Culicoides* cells more permissive to SBV replication.

These results suggested that polyclonal antibodies that we developed will allow us to establish kinetic of N nucleoprotein production, to localize viral factories and to compare virus processing and egress in both cell lines by confocal analyses of immunofluorescence assays.

SBV adaptation in KC cells long term infection

As amplifications ratios were poor in KC cells compared with those obtained in BHK-21, we postulated that it could be due to RNAi response induced by viral infection. We supposed that this RNA interference could exert a selective pressure on the virus and lead to the selection of a mutant adapted to RNAi which present a higher rate of replication. To test this hypothesis, we performed long term infection in KC cells and tested viral amplification rate by RT-RT one step qPCR for SBV S and L segments and by titration in BHK-21 cells. We also sequenced S segment and hypervariable region (M segment) to study mutation accumulation during adaptation to KC cells.

Results showed that SBV started to amplify after passage 5 in KC cells until CT that had never been observed previously as assessed by RT-RT one step qPCR. This change in the rate of growth suggested that viral adaptation occurred. Sequence analyses demonstrated that three mutations appeared between passages 1 and 5 in the hvr and were maintained through cellular passages. These mutations could be responsible of viral adaptation to KC cells. Titration on BHK-21 demonstrated that adapted virus were still infectious in BHK-21 suggesting that adaptation did not impair the capacity to interact and to replicate in mammalian cells.

The sequences obtained for small segment and hvr region were aligned with other SBV sequences from Fischer *et al.*, 2013 and Coupeau *et al.*, 2013 papers. Mutation at position 661 observed in KC cells was also found in BH231/12-1 and BH250/12-2 isolates. Impact of these mutations on amino acid sequence was also investigated for SBV sequences of these last papers. For sequence BH281/12-5, an amino acid change from N to I was observed at the position 880 and was attributed to mutations at position 2662 and 2663. These results supported the hypothesis that the region on M segment we sequenced is hyper variable and may have an important role on SBV adaptation to cell line. Moreover, the number of mutations (on small segment and hvr) after nine successive passages in KC cell was lower than the number of mutations after 10 passages in BHK-21 cells (ex: 3 for Na-1 and 10 for Na-2) (Coupeau *et al.*, 2013).

No mutations were found in the S segment for any cellular passages. This result is in agreement with data about Simbuviruses S segment. Indeed, according to Kobayashi *et al.*, (2007), S segment is more conserved than M segment for Simbuviruses and it was confirmed for SBV in the study of Coupeau *et al.*, 2013. However, Bunyamwera NSs protein has been showed to be important in certain mosquito cells for viral replication, suggesting the need of a complementarity between NSs and cellular component to ensure viral replication. NSs has been suggested to be crucial for efficient infection in certain mosquito cells and live mosquitoes (Szemiel *et al.*, 2012). Indeed, Bunyamwera lacking NSs had more difficulty in overcoming midgut escape barrier mosquitoes. (To be successfully transmitted by a vector to a vertebrate, an arbovirus should disseminate in secondary organs as muscles, haemolymph, salivary glands after replication in the midgut). It was demonstrated that the lack of NSs delayed the progress of infection suggesting that NSs is required for efficient replication and spread in mosquitoes.

Therefore, it was reasonable to expect mutation in the ORF of SBV NSs after several passages in KC cells which lead to high replication rate. As no mutations were observed in the NSs while viral adaptation occurred, two hypotheses were mounted. Firstly, we postulated that cellular component in KC cells which could interact with NSs have underwent modification that allowed viral replication in a high rate in KC cells. To test this hypothesis, we should infect the initial stock of KC cells with adapted virus and monitored viral amplification by quantitative PCR and titration. Secondly, we postulated that another viral component should have been modified and played a role in viral adaptation to KC cells. Therefore, full genome sequencing is necessary to investigate on the origin of SBV adaptation to KC cells.

On the other hand, comparative SBV sequence analysis in the study of Fischer *et al* (2013) revealed a very high stability for the nucleoprotein ORF which is consistent with our observations. Nucleoprotein high stability could be due to its important role in RNA encapsidation. Indeed, it has been showed that Bunyaviruses avoid dsRNA-based RNAi response by coating their RNA segments with nucleoprotein (Newton *et al* 1981, cited in Szemiel *et al.*, 2012). The fact that we observed no mutations in nucleoprotein while viral adaptation to KC cells occurred supported two hypotheses. The first one is that another viral protein than the nucleoprotein is implicated in RNAi avoiding in KC cells infected with SBV. The second hypothesis is that the observed viral adaptation is not related to RNAi escape suggesting that a mechanism which limited viral replication has been abolished. This mechanism could be the sequestration of important proteins for viral replication as nucleoprotein and L polymerase. Indeed, this phenomenon was observed by Lopez Montero

et al., (2011) in mosquito cells infected with Bunyamwera. Therefore, to investigate on this hypothesis, immuno-staining of cellular and viral components in infected cells are needed. They will be performed with KC cells adapted SBV viral strain.

Globally, the low number of mutations observed in KC cells long term infection by SBV could be due to vector-dependent characteristic of SBV. *Indeed, arboviruses having an alternate two-host 'life cycle' are suggested to be more stable than vector-independent viruses (Fischer et al., 2013). Genomic evolution is suggested to be slower if a virus has to adapt to two hosts (Moutailler et al., 2011).* As SBV is transmitted by arthropod vectors, the virus undergoes two processes of replication cycles: one in the arthropod vector and the other in the vertebrate host, and selective pressures may act during both steps. Fischer *et al* wondered whether sequence divergence is related to the mammalian or arthropod portion of the virus life cycle. As the number of mutations is lower after successive passages in KC cell compare with successive passage in BHK-21 cells (Coupeau *et al.*, 2013) it suggests that SBV is more adapted to KC cells than to BHK-21 cells.

The relevance and the impact of the three mutations we have found in adapted virus to KC cells need to be confirmed by reverse genetic system. However, the hypothesis that these three mutations are implied in viral adaptation is still plausible. Indeed, it has been demonstrated that a low number of mutations could lead to viral adaptation as it was explained for Encephalitis equine virus (Anischenko *et al*, 2006). Venezuelan equine encephalitis virus (VEEV) is an arbovirus transmitted by mosquitoes that initially circulated among rodents. Only one amino acid change was sufficient to evolve from an enzootic equine avirulent strain circulating in rodents to an epidemic strain responsible of neurological disease in horses.

Conclusion and perspectives

Our first objective was to characterize SBV interaction with KC and BHK-21 cells. SBV rate of growth, the parameters influencing it and cytopathic effects in both cell lines were studied. Results showed that SBV amplification was possible in KC cells derived from *Culicoides sonorensis* supporting studies demonstrating *Culicoides* competence for SBV. However, it was observed that the amplification ratios in KC cells were lower than in BHK-21 cells. We postulated that this difference could be due to the distinct cell response between mammals and insects. Specifically RNAi pathway in KC cells seems to be implicated in maintaining SBV amplification in a rate that is not harmful for the vector. Another hypothesis could be the sequestration of important viral proteins or the replication limited to the peripheral Golgi stacks in KC cells. Moreover, in *Culicoides* cells, no cytopathology was observed while in mammalian cells, infection led to cell lysis and death. A release method that did not involve insect cell membrane rupturing could explain why SBV replication did not killed *Culicoides* cells. To investigate on these two last hypotheses, SBV course and interaction with cellular component of SBV in KC and BHK-21 should be studied.

The second objective was to study by immunofluorescence if SBV replication in different part of the Golgi, trapping of viral protein and the way of releasing virions could affect cells viability and viral rate of growth. For that purpose, polyclonal antibodies directed against SBV N nucleoprotein (a protein highly expressed in infected cells) were developed. Production of recombinant SBV N nucleoprotein was concluding as shown by mass

spectrometry analysis, recombinant proteins were pure. Immunization of rabbits with recombinant nucleoprotein give rise to antibodies directed against recombinant SBV N nucleoprotein. Analysis of antibodies by ELISA, Western blot and immunofluorescence assay showed coherent results. Indeed, these three assays suggested that antibodies were specific to recombinant and native SBV N nucleoprotein and specifically recognized infected cells. Therefore, antibodies developed in this work will be used to follow SBV course inside the cell by immunofluorescence assay implying multiple cellular staining. This will allow us to investigate on virions egress, localization of viral factories and viral replication kinetics.

As revealed by immunofluorescence and western blot assays, viral amplification was initially very low in KC cells. Therefore, the third objective was to develop a viral strain adapted to KC cells presenting a higher amplification ratio. To this end, long term infection in KC cells was performed and SBV amplification was monitored by quantitative PCR and titration. After passage 5, viral adaptation occurred. To investigate on the origin of this adaptation, SBV small segment and hyper-variable region (part of medium segment) were sequenced. Three mutations appeared between passage 1 and 5 and were maintained through successive cellular passages. The next step will be to infect BHK-21 and KC cells with KC cells adapted SBV to study differences in rate of growth and in cytopathic effects with initial SBV and perform immunofluorescence in both cells lines. Moreover, complete genome sequencing and reverse genetic experiments are needed to assess if these mutations are implied in viral adaptation to KC cells. These further experiments will allow investigation on the hypothesis of RNAi response that limits replication in KC cells. Another way of investigating on RNAi effect on SBV genetic sequence and amplification ratio would be to study SBV growth in cells deprived of RNAi response as C6/36 mosquito cells.

To assess if the sequestration of important viral proteins also play a role in limiting SBV replication in KC cells, multiple cellular components (Golgi, ER,...) and monoclonal antibodies directed against SBV proteins are needed. These lasts will allow the identification of cellular components important for viral replication and to investigate on differences in CPE and viral rate of growth observed between KC and BHK-21 cells.

Otherwise, SBV has been related to Shamonda (SHAV) and Sathuperi (SATV) and is suggested to be a reassortant of these both viruses. As SBV has a two host alternate life cycle, the recombination processes which lead to its formation may have appeared in vertebrate host or in insect vector. To investigate on SBV origin, co-infection experiments with SHAV and SATV should be performed in KC and BHK-21 cells. These experiments have been already performed in BHK-21 cells. However, poor amplification ratio of SATV and SHAV in KC cells limited study of progeny viruses in this cell line. Therefore, investigation of SBV adaptation to KC cells will allow us to find a solution by shutting of RNAi response or in developing SATV and SHAV adapted to KC cells by long term infections. Progeny virions produced by co-infection in KC cells will be genotyped by HRMA (High-resolution melting analysis), a rapid and high throughput method that we previously developed. Results of further co-infection experiments will be compared with those obtained in BHK-21 cells and will allow us to investigate on SBV origin.

It is also important to keep in mind that replication rates *in vitro* do not necessarily reflect replication rates *in vivo*. Yet, it has been demonstrated that digestive enzymes within the insect gut can affect the infectivity of viruses in vectors (Mertens *et al*, 1987 cited in Mellor *et al*, 2000). Indeed, treatment of BTV (bluetongue virus) with chymotrypsin and trypsin gave rise to infectious subviral particles that are highly orally infective for *Culicoides*

vectors. Therefore, it could be interesting to treat SBV with such proteases before infection in KC cells.

The next step after *in vitro* studies will be the establishment of *Culicoides* colonies to study infection *in vivo* with SHAV/SATV/ SBV and to investigate on the relation of these three related Simbuviruses within their vectors and to investigate on SBV origin.

Bibliography

Bibliography

- Anishchenko M, Bowen RA, Paessler S, Austgen L, Greene IP, Weaver SC. (2006)** Venezuelan encephalitis emergence mediated by a phylogenetically predicted viral mutation. *Proc Natl Acad Sci U S A* 103(13) : 4994-9
- Balenghien T and Delécolle J. (2009)** Les Culicoides mouchérons vecteurs du virus de la fièvre catarrhale ovine. *Insectes* 25 n°154
- Barry G, Varela M, Ratnier M, Blomström AL, Caporale M, Seehusen F, Hahn K, Schnettler E, Baumgärtner W, Kohl A, Palmarini M. (2014)** NSs protein of Schmallenberg virus counteracts the antiviral response of the cell by inhibiting its transcriptional machinery. *J Gen Virol*. 95(Pt 8) : 1640-6
- Beer M, Conraths FJ, WH VDP (2012)** 'Schmallenberg virus' - a novel orthobunyavirus emerging in Europe. *Epidemiol Infect* : 1-8
- Bilk S, Schulze C, Fischer M, Beer M, Hlinak A, Hoffmann B. (2012)** Organ distribution of Schmallenberg virus RNA in malformed newborns. *Vet Microbiol* 159(1-2) : 236-8
- Borucki MK, Kempf BJ, Blitvich BJ, Blair CD, Beaty BJ. (2002)** La Crosse virus: replication in vertebrate and invertebrate hosts. *Microbes Infect* 4 : 341-350
- Briese T, Calisher CH, Higgs S. (2013)** Viruses of the family Bunyaviridae: are all available isolates reassortants? *Virology* 446(1-2) : 207-16
- Burgin LE, Gloster J, Sanders C, Mellor PS, Gubbins S, Carpenter S. (2013)** Investigating Incursions of Bluetongue Virus Using a Model of Long-Distance Culicoides Biting Midge Dispersal. *Transbound Emerg Dis* 60 : 263-272
- Carpenter S, Groschup M, Garros C, Felipe- Bauer M, Purse B. (2013)** Culicoides biting midges, arboviruses and public health in Europe. *Antiviral Res* 100(1) : 102-13
- Claine F, Coupeau D, Wiggers L, Muylkens B, Kirschvink N. (2013)** Schmallenberg virus among female lambs, Belgium, 2012. *Emerg Infect Dis* 19(7): 1115-1117
- Coupeau D, Claine F, Wiggers L, Kirschvink N, Muylkens B. a (2013)** In vivo and in vitro identification of a hypervariable region in Schmallenberg virus. *J Gen Virol* 94(6) : 1168-1174
- Coupeau D, Claine F, Wiggers L, Martin B, Kirschvink N, Muylkens B. b (2013)** Characterization of messenger RNA termini in Schmallenberg virus and related Simbuviruses *J Gen Virol* 94 (Pt 11) : 2399-405
- Craig GB , Hickey WA. (1967)** Current status of the formal genetics of *Aedes aegypti*. *Bull World Health Organ* 36(4): 559-562
- De Regge N, Deblauwe I, De Deken R, Vantieghem P, Madder M, Geysen D, Smeets F, Losson B, van den Berg T, Cay AB. (2012)** Detection of Schmallenberg virus in different Culicoides spp. by real-time RT-PCR. *Transbound Emerg Dis* 59(6) : 471-5

Doceul V, Lara E, Sailleau C, Belbis G, Richardson J, Bréard E, Viarouge C, Dominguez M, Hendriks P, Calavas D, Desprat A, Languille J, Comtet L, Pourquier P, Eléouët JF, Delmas B, Marianneau P, Vitour D, Zientara S. (2013) Epidemiology, molecular virology and diagnostics of Schmallenberg virus, an emerging orthobunyavirus in Europe. *Vet Res* 15: 44-31

Domingo E. (2010) Mechanisms of viral emergence. *Vet. Res* 41(6):38

Dong H, Li P, Elliott RM, Dong C. (2013) Structure of Schmallenberg orthobunyavirus nucleoprotein suggests a novel mechanism of genome encapsidation. *J Virol* 87(10) : 5593-601

European Food Safety Authority (EFSA) (2013) "Schmallenberg" virus: analysis of the epidemiological data. EN-429 22 pp

Elbers AR, Meiswinkel R, van Weezep E, Sloet van Oldruitenborgh-Oosterbaan MM, Kooi EA. (2013) Schmallenberg virus in *Culicoides* spp. biting midges, the Netherlands, 2011. *Emerg Infect Dis.* 19(1): 106-9

Elliott Richard. (2014) Orthobunyaviruses: recent genetic and structural insights *Nature Reviews Microbiology* 12: 673–685

Endres M, Griot C, Gonzalez-Scarano F, Nathanson N. (1991) Neuroattenuation of an avirulent bunyavirus variant maps to the LRNA segment. *Virology* 65 : 5465–5470

Fields Virology by Lippincott, Williams, Wilkins. (2007) Volume 1 Fifth Edition: 3177

Fischer M, Hoffmann B, Goller KV, Höper D, Wernike K, Beer M. (2013) A mutation 'hot spot' in the Schmallenberg virus M segment. *J Gen Virol* 94(Pt 6) : 1161-7

Garigliany MM, Bayrou C, Kleijnen D, Cassart D, Jolly S, Linden A, Desmecht D. (2012) Schmallenberg virus: a new Shamonda/Sathuperi-like virus on the rise in Europe. *Antiviral Res* 95(2) : 82-87

Hardy J. (1988) Susceptibility and resistance of vector mosquitoes. *Arboviruses: Epidemiology and Ecology* Vol I : 87–126

Hayashi K, Suzuki H, Makino Y. (1978) Notes on the transoceanic insects captured on the East China Sea in 1976–1978. *Trop Med* 21 : 1–10

He CQ, Ding NZ. (2012) Discovery of severe fever with thrombocytopenia syndrome bunyavirus strains originating from intragenic recombination. *J Virol* 86(22) : 12426-30

Hepojoki J, Strandin T, Wang H, Vapalahti O, Vaheri A, Lankinen H. (2010) Cytoplasmic tails of hantavirus glycoproteins interact with the nucleocapsid protein. *J Gen Virol* 91 : 2341–2350

Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, Schirrmeier H, Eschbaumer M, Goller K, Wernike K, Fischer M, Breithaupt A, Mettenleiter T, Beer M. (2012) Novel orthobunyavirus in Cattle, Europe, 2011. *Emerg Infect Dis* 18(3) : 469-472

Holmes E. (2006) The evolution of viral emergence. *Proc. Natl. Acad. Sci. USA* 103(13) : 4803-4804

Houk E, Kramer L, Hardy J and Presser S. (1986). An intraspecific mosquito model for the mesenteron infection barrier to western equine encephalomyelitis virus (*Culex tarsalis* and *Culex pipiens*). *American Journal of Tropical Medicine and Hygiene* 35 : 632-641

Iroegbu CU, Pringle CR. (1981) Genetic interactions among viruses of the Bunyamwera complex. *J Virol* 37(1) : 383-94

Jennings D and Mellor P. (1987) Variation in the responses of *Culicoides variipennis* (Diptera: Ceratopogonidae) to oral infection with bluetongue virus. *Archives of Virology* 95 p. 177-182.

Jones R and Foster N. (1974) Oral infection of *Culicoides variipennis* with bluetongue virus: development of susceptible and resistant lines from a colony population. *Journal of Medical Entomology* 11: 316-323

Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, et al. (2008) Global trends in emerging infectious diseases. *Nature* 451 : 990-993

Knipe D, Howley P. (2007) Fields Virology fifth edition. Volume one : 1-3177

Kobayashi T, Yanase T, Yamakawa M, Kato T, Yoshida K, Tsuda T. (2007) Genetic diversity and reassortments among Akabane virus field isolates. *Virus Res* 130, : 162-171.

Koenraadt CJ, Balenghien T, Carpenter S, Ducheyne E, Elbers AR, Fife M, Garros C, Ibáñez-Justicia A, Kampen H, Kormelink RJ, Losson B, van der Poel WH, De Regge N, van Rijn PA, Sanders C, Schaffner F, Sloet van Oldruitenborgh-Oosterbaan MM, Takken W, Werner D, Seelig F. (2014) Bluetongue, Schmallenberg - what is next? *Culicoides*-borne viral diseases in the 21st Century. *BMC Vet Res*.10 :77

Kraatz F, Wernike K, Hechinger S, König P, Granzow H, Reimann I, Beer M. (2014) Deletion mutants of Schmallenberg virus are avirulent and protect from virus challenge. *J Virol* pii: JVI.02729-14

Lazaro E, Escarmis C, Perez-Mercader J, Manrubia S, Domingo E. (2003) Resistance of virus to extinction on bottleneck passages: study of a decaying and fluctuating pattern of fitness loss, *Proc Natl Acad Sci USA* 100 : 10830-10835

Lazutka J, Zvirbliene A, Dalgediene I, Petraityte-Burneikiene R, Spakova A, Sereika V, Lelesius R, Wernike K, Beer M, Sasnauskas K. (2014) Generation of recombinant schmallenberg virus nucleocapsid protein in yeast and development of virus-specific monoclonal antibodies. *J Immunol Res* 2014 : 1-8

Lillie TH, Marquard WC, Jones RH. (1981) The flight range of *Culicoides variipennis* (Diptera: Ceratopogonidae) *Can. Entomol.* 133 : 419-42.6

Linden A, Desmecht D, Volpe R, Wirtgen M, Gregoire F, Pirson J, Paternostre J, Kleijnen D, Schirrmeier H, Beer M, Garigliany MM. (2012) Epizootic spread of Schmallenberg virus among wild cervids, Belgium, Fall 2011. *Emerg Infect Dis* 18(12) : 2006-2008

López-Montero N, Risco C. (2011) Self-protection and survival of arbovirus-infected mosquito cells. *Cell Microbiol* 13(2) : 300-15

Martinelle, Dal Pozzo F, Kirschvink N, De La Grandière M.A, Thiry E, Saegerman C. (2012) Le virus Schmallenberg ou l'émergence du premier Orthobunyavirus du séro groupe Simbu en Europe. *Ann. Méd. Vét* 156 : 7- 24

Mayhew I. (1984) Neuromuscular arthrogryposis multiplex congenita in a thoroughbred foal. *Vet Pathol* 21: 187-192.

Mecham JO. (2006) Detection and titration of bluetongue virus in Culicoides insect cell culture by an antigen-capture enzyme-linked immunosorbent assay. *J Virol Methods* 135(2) : 269-71

Meiswinkel R. (1989) Afrotropical *Culicoides*: a redescription of *C. (Avaritia) imicola* Kieffer, 1913 (Diptera: Ceratopogonidae) with description of the closely allied *C. (A.) bolitinos* sp. nov. reared from the dung of the African buffalo, blue wildebeest and cattle in South Africa. *Onderstepoort J Vet Res* 56 :23-39.

Meiswinkel R, Nevill EM, Venter GJ. (1994) Vectors: Culicoides spp. *See Ref* 36 :68-89

Mellor P. (2000) Replication of arboviruses in insect vectors. *J Comp Path* 123 : 231-247

Mellor P, Boorman J, Baylis M. (2000) Culicoides biting midges: their role as arbovirus vectors. *Annu Rev Entomol* 45: 307-40.

Morazzani EM, Wiley MR, Murreddu MG, Adelman ZN, Myles KM. (2012) Production of virus-derived ping-pong-dependant piRNA-like small RNAs in the mosquito soma. *PLoS Pathogens* 8: e1002470

Mullens BA, Gerry AC, Lysyk TJ, Schmidtman ET. (2004) Environmental effects on vector competence and virogenesis of bluetongue virus in Culicoides: interpreting laboratory data in a field context. *Vet Ital* (3):160-166

Muskens J, Smolenaars AJ, van der Poel WH, Mars MH, Van Wuijckhuise L, Holzhauser M, Van Weering H, Kock P. (2012) Diarrhea and loss of production on Dutch dairy farms caused by the Schmallenberg virus. *Annu Rev Entomol.* 45 : 307-40

Nayduch D, Cohnstaedt LW, Saski C, Lawson D, Kersey P, Fife M, Carpenter S. (2014) Studying Culicoides vectors of BTV in the post-genomic era: resources, bottlenecks to progress and future directions. *Virus Res* 182 : 43-9.

Newton SE, Short NJ, Dalgarno L. (1981) Bunyamwera virus replication in cultured Aedes albopictus (mosquito) cells: establishment of a persistent viral infection. *J virol* 38 : 1015-1024

Plassmeyer ML, Soldan SS, Stachelek KM, Roth SM, Martin-Garcia J, Gonzalez-Scarano F. (2007) Mutagenesis of the La Crosse Virus glycoprotein supports a role for Gc (1066–1087) as the fusion peptide. *Virology* 358 : 273–282

Rasmussen LD, Kristensen B, Kirkeby C, Rasmussen TB, Belsham GJ, Bødker R, Bøtner A. (2012) Culicoids as vectors of Schmallenberg virus. *Emerg Infect Dis* 18(7) : 1204-6

Rees J, Moniatte M and Bulet P. (1997) Novel antibacterial peptides isolated from a European bumblebee, *Bombus pascuorum* (Hymenoptera, Apo-idea). *Insect Biochemistry and Molecular Biology* 27 : 413–422

Reusken C, van den Wijngaard C, van Beek P, Beer M, Bouwstra R, Godeke GJ, Isken L, van den Kerkhof H, van Pelt W, van der Poel W, Reimerink J, Schielen P, Schmidt-Chanasit J, Vellema P, de Vries A, Wouters I, Koopmans M. (2012) Lack of evidence for zoonotic transmission of Schmallenberg virus. *Emerg Infect Dis* 18(11) : 1746-54

Rodriguez LL, Owens JH, Peters CJ, Nichol ST. (1998) Genetic reassortment among viruses causing hantavirus pulmonary syndrome. *Virology* 1;242(1) : 99-106

Salanueva, I, Novoa R, Cabezas P, López-Iglesias C, Carrascosa J, Elliott R, and Risco C. (2003) Polymorphism and structural maturation of Bunyamwera virus in Golgi and post-Golgi compartments. *J Virol* 77 : 1368–1381

Sanjuan R, Nebot M, Chirico N, Mansky L, Belshaw R. (2010) Viral Mutation Rates *J Virol* 84(19) : 9733–9748

Sedda L, Rogers DJ. (2013) The influence of the wind in the Schmallenberg virus outbreak in Europe. *Sci Rep* 3:3361.

Shi X, Kohl A, Li P, Elliott RM. (2007) Role of the cytoplasmic tail domains of Bunyamwera orthobunyavirus glycoproteins Gn and Gc in virus assembly and morphogenesis. *J Virol*. 81(18) : 10151-60

Shi X, Kohl A, Léonard VH, Li P, McLees A, Elliott RM. (2006) Requirement of the N-terminal region of orthobunyavirus nonstructural protein NSm for virus assembly and morphogenesis. *J Virol* 80(16) : 8089-99

Szemiel A, Failloux A, Elliott R. (2012) Role of Bunamwera orthobunyavirus NSs protein in infection of mosquito cells. *PLoS Neg Trop Dis* 6(9) e1823

Tabachnick W. (1996) *Culicoides variipennis*, and bluetongue virus epidemiology in the United States. *Ann rev entomol* 41 : 23-43

Takahashi, M. (1982) Differential transmission efficiency for Japanese encephalitis among colonized strains of *Culex tritaeniorhynchus*. *Japanese Journal of Sanitary Zoology*, 3 : 325–333

Tarlinton R, Daly J, Dunham S, Kydd J. (2012) The challenge of Schmallenberg virus emergence in Europe. *Vet J* 194(1): 10-8

Truant AL, Hallum JV.(1977) A persistent infection of baby hamster kidney-21 cells with mumps virus and the role of temperature-sensitive variants. *J Med Virol* 1(1): 49-67

Varela M, Schnettler E, Caporale M, Murgia C, Barry G, McFarlane M, McGregor E, Piras IM, Shaw A, Lamm C, Janowicz A, Beer M, Glass M, Herder V, Hahn K, Baumgärtner W, Kohl A, Palmarini M. (2013) Schmallenberg virus pathogenesis, tropism and interaction with the innate immune system of the host. *PLoS Pathog* 9(1):e1003133

Veronesi E, Henstock M, Gubbins S, Batten C, Manley R, Barber J, Hoffmann B, Beer M, Attoui H, Mertens PP, Carpenter S. (2013) Implicating Culicoides biting midges as vectors of Schmallenberg virus using semi-quantitative RT-PCR. *PLoS One* 8(3):e57747

Walter CT, Barr JN. (2011) Recent advances in the molecular and cellular biology of bunyaviruses. *J Gen Virol* 92 : 2467–2484

Walzer B. (2009) Les insectes du genre culicoides vecteurs de maladies animales. Thèse pour le doctorat : école nationale vétérinaire d'Alfort : 1-220

Wensman JJ, Blomqvist G, Hjort M, Holst BS. (2013) Presence of antibodies to Schmallenberg virus in a dog in Sweden. *J Clin Microbiol* 51(8) : 2802-2803

Wernike K, Jöst H, Becker N, Schmidt-Chanasit J, Beer M. (2014) Lack of evidence for the presence of Schmallenberg virus in mosquitoes in Germany, 2011. *Parasit Vectors* 29 (7): 402

Wessner D. (2010) The origins of viruses. *Nature Education* 3(9):37

Yanase T, Aizawa M, Kato T, Yamakawa M, Shirafuji H, Tsuda T. (2010) Genetic characterization of Aino and Peaton virus field isolates reveals a genetic reassortment between these viruses in nature. *Virus Res* 153 : 1–7

Yanase T, Kato T, Aizawa M, Shuto Y, Shirafuji H, Yamakawa M, Tsuda T. (2012) Genetic reassortment between Sathuperi and Shamonda viruses of the genus Orthobunyavirus in nature: implications for their genetic relationship to Schmallenberg virus. *Arch Virol* 157(8) : 1611-6

Supplementary data

Supplementary data

Table 1: SBV amplification ratio over the time during growth kinetics in KC and BHK-21 cells (refers to Figure 15)

a		b		c		d	
Time	Ratio	Time	Ratio	Time	Ratio	Time	Ratio
8	1	8	1	2	1	2	1
28	3807,46766	28	11,388787	24	10,2847558	8	2,39647413
40	8360,87429	40	0,67975963	48	10,2847558	24	3,44927686
		48	23,0833467			32	6,17698506
						48	9,5623123

Table 2: Optical density registered during ELISA assay on several rabbit serum dilutions

	0,0005	0,00025	0,000125	0,0000625	0,00003125	0,000015625	7,8125E-06	3,90625E-06	1,95313E-06
L 295-1	0,9765	0,5955	0,3295	0,1945	0,1195	0,0775	0,059	0,0485	0,053
L 295-2	2,9385	2,395	1,7175	1,194	0,7035	0,478	0,257	0,156	0,109
L 296-1	1,2095	0,721	0,4235	0,2385	0,14	0,092	0,0665	0,054	0,0495
L 296-2	3,134	3,0095	2,756	2,385	1,941	1,366	0,828	0,5065	0,2945
L 281	0,048	0,0545	0,043	0,045	0,043	0,043	0,0425	0,0435	0,0435

Table 3: CT value obtained by qPCR one step on SBV L and S segment for three cellular conditions (A100%, B100% and B66%) through cellular passage (3 to 9) of long term infected KC cells.

qRTPCR one step L segment

	3	4	5	6	7	8	9
A 100%	30,12	32,76	32,42	26,24	22,1	13,61	16,68
B 100%	28,8	32,86	31,56	27,59	26,75	20,51	18,67
B66%	30,92	36,06	36,16	NA	NA	35	33,16

qRTPCR one step S segment

	3	4	5	6	7	8	9
A 100% S	25,22	29,48	29,5	22,71	18,62	10,89	11,34
B 100% S	24,85	28,61	27,6	23,88	22,31	17,02	15,1
B66% S	26,17	30,67	31,49	32,25	34,14	30,65	29,08

Table 4: SBV titer (TCID50) corresponding to cellular suspension sampled in KC long term infection experiment.

	3	4	5	6	7	8	9	10	11
TCID50	0	0	2,00E+02	2,00E+02	9,28E+03	4,31E+05	1,18E+06	2,00E+05	4,31E+05
Log10 (TCID50)	/	/	2,30103	2,30103	3,96754798	5,63447727	6,07188201	5,30103	5,63447727